JOURNAL OF BACTERIOLOGY, Aug., 1965
Vol. 90, No. 2

Printed in U.S.A.

Formation of Ribosomes from Precursor Ribonucleoprotein Particles

PAUL S. SYPHERD[1]

Department of Biology, University of California, San Diego, California, and Department of Microbiology, University of Illinois, Urbana, Illinois

Received for publication 31 March 1965

ABSTRACT

SYPHERD, PAUL S. (University of California, San Diego). Formation of ribosomes from precursor ribonucleoprotein particles. J. Bacteriol. **90**:411–417. 1965.—Amino acid deprivation of a "relaxed" mutant leads to the accumulation of ribonucleoprotein particles. The possibility that these particles are equivalent to normal ribosome precursors was investigated. This was accomplished by determining the fate of such particles after the termination of starvation, and subsequent growth. It was found that both the nucleic acid and the protein moieties of the "relaxed" particles were converted to mature ribosomes during the recovery phase. From kinetic measurements with "relaxed" particles and normal ribosome precursors, it was shown that they were metabolically indistinguishable. It was concluded that the ribonucleoprotein "relaxed" particles accumulated by the relaxed mutant are immature ribosomes, and equivalent to ribosome precursors which occur under normal growth conditions.

A previous report (Sypherd, 1965) has shown that when a relaxed (RC^{rel}) mutant of *Escherichia coli* is deprived of an essential amino acid, ribonucleoprotein (RNP) particles accumulate as the result of the overproduction of ribonucleic acid (RNA). As a group these particles, with nominal *S*-values of 20, 30, and 45, were shown to be less than 30% protein. A similar protein content has been reported for RNP which accumulates in bacteria treated with chloramphenicol (Nomura and Watson, 1959). Both relaxed starvation and chloramphenicol treatment are characterized by the continued synthesis of nucleic acid in the absence of net protein synthesis.

The suggestion has been made by others that the RNA formed during relaxed starvation (Dagley, Turnock, and Wild, 1962) and during chloramphenicol treatment (Nomura and Watson, 1959) is present in a form similar to natural precursors of ribosomes. With the aim of studying the biochemical events in ribosome formation, an attempt was made to ascertain the physiological significance of the RNP particles accumulated during relaxed starvation. The possibility that "relaxed particles" are natural intermediates in the formation of ribosomes has been investigated by studying precursor relationships between the particles and mature ribosomes. This was accomplished by determining the fate of the relaxed RNP particles in cells recovering from amino acid deprivation. During the course of the experiments, Nakada, Anderson, and Magasanik (1964) reported on the fate of the nucleic acid formed during relaxed starvation. Their findings have been verified and extended to include the entire RNP particle.

MATERIALS AND METHODS

Bacterial strains, media, and biochemical methods are described in the accompanying paper (Sypherd, 1965).

RESULTS

The characteristics of general RNA and protein synthesis during amino acid starvation and subsequent growth are shown in Fig. 1. After termination of the starvation period, the culture experiences a lag in the resumption of growth prior to attainment of exponential growth. In contradistinction to the relaxed mutant, amino acid auxotrophs which carry the RC allele in the wild-type (stringent) state do not continue to synthesize RNA when deprived of the required amino acid. In addition, there was immediate resumption of growth after the addition of amino acid to the stringent auxotroph (Fig. 2).

It was not immediately clear why amino acid deprivation of the RC^{rel} organism, and concomitant RNA accumulation, induced the lag in growth after the termination of starvation. However, the lag seemed to be related to the accumulation of RNA. This was shown by use of a

[1] Present address: Department of Microbiology, University of Illinois, Urbana.

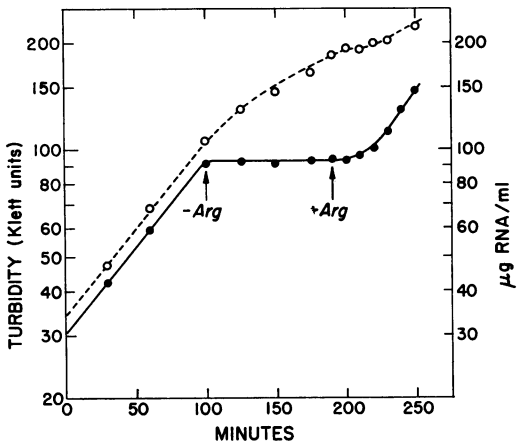

FIG. 1. *Synthesis of RNA during amino acid deprivation of RC^{rel} mutant HAR. An exponentially growing culture was filtered onto a Millipore filter, size HA, and washed with 10 ml of distilled water. The cells were resuspended in medium lacking arginine. Recovery of cells from the filter was virtually complete. Cessation of growth occurred immediately after resuspension. Starvation was terminated by adding 50 μg/ml of L-arginine as indicated. Symbols: RNA (○), turbidity (●).*

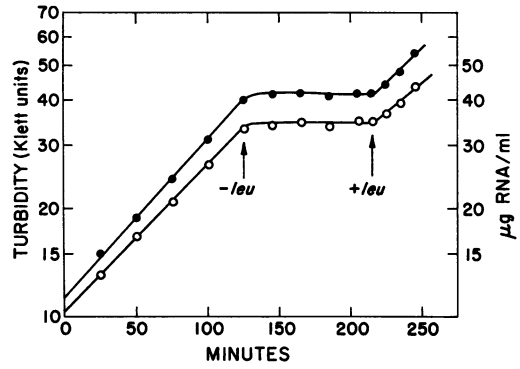

FIG. 2. *Cessation of RNA synthesis during amino acid deprivation of an RC^{stringent} organism. Experimental procedure and symbols as in Fig. 1.*

methionine-uracil double auxotroph which carried the RC^{rel} allele. When this organism was deprived of the required amino acid, RNA accumulation and the growth lag after addition of methionine were evident (Table 1). However, when both methionine and uracil were removed from the medium, there was neither RNA accumulation nor the subsequent growth lag after the termination of starvation.

The accumulation of RNA by the relaxed mutant may have even more serious effects on subsequent growth. If the cultures are allowed to triple their usual complement of RNA without concomitant protein synthesis, the subsequent growth lag may last for several hours. Unsuccessful attempts were made to reverse the lag with various nutrients, including amino acid and purine-pyrimidine mixtures. The significance of the growth lag after relaxed starvation is not presently appreciated. Though it may be postulated that the accumulated RNA has some deleterious effect on the physiological well-being of the cells, it is difficult to visualize what this effect may be in biochemical terms.

The growth-lag period provided an opportunity to study the fate of the RNA which was accumulated during amino acid deprivation. The stability of relaxed RNA after termination of the starvation period was examined. Nucleic acids formed

TABLE 1. *Characteristics of RNA accumulation and growth lag in a methionine-uracil auxotroph with the RC^{rel} allele*

Time	Period	Methionine-starved		Methionine-uracil-starved	
		Protein	RNA	Protein	RNA
min					
0	Starvation	210*	104	181	91
15		213	120	183	91
30		208	135	182	93
60		212	148	186	90
90		214	164	184	91
10	Recovery	214	170	193	96
20		215	176	205	104
30		222	186	215	118

* Expressed as micrograms per milliliter of culture.

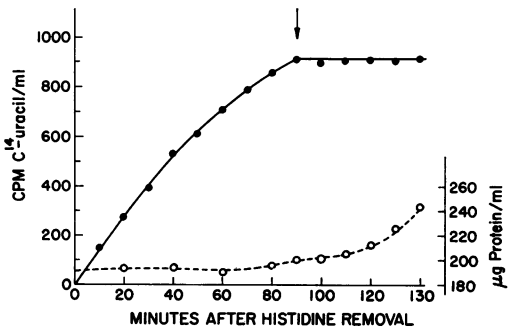

FIG. 3. *Incorporation of uracil-C^{14} into nucleic acid during histidine deprivation of HAR (●). At the time indicated by the arrow, the starved culture was filtered from the radioactive medium, washed on the filter with distilled water, and resuspended in complete medium containing 50 μg/ml of histidine and 200 μg/ml of uracil-C^{12}. Protein content of the culture is shown on the right ordinate (○).*

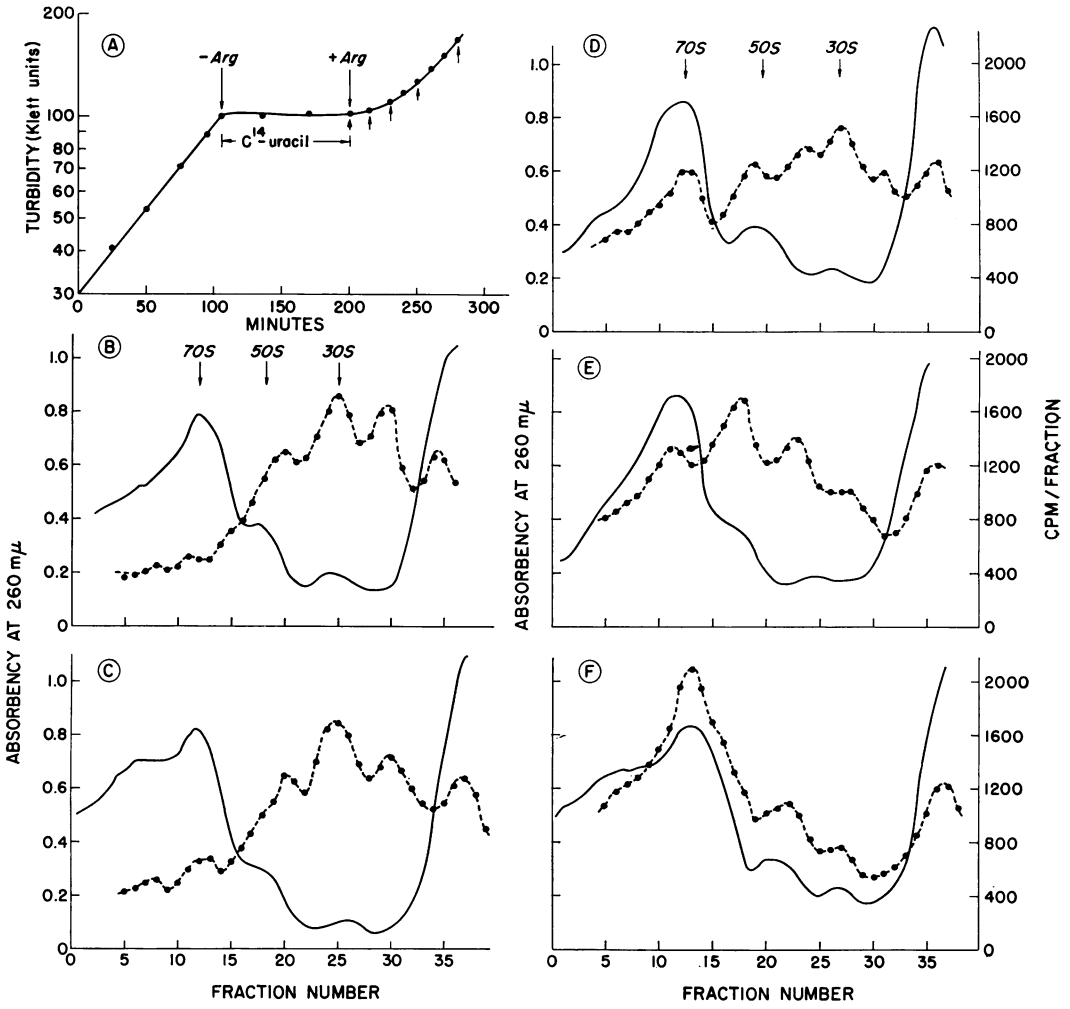

FIG. 4. *Conversion of relaxed particles to ribosomes after termination of starvation. (A) Turbidity readings during the course of the experiment. Samples taken at times indicated by the arrows were analyzed in (B) through (F). (B) Distribution of relaxed material immediately after arginine addition. The distribution of this material is shown at subsequent times of 15 min (C), 30 min (D), 50 min (E), and 80 min (F) after arginine addition. Solid lines show the absorbancy profile of the extract made with a 25-fold excess of carrier, nonstarved cells. Relaxed RNA is shown by the broken lines (uracil-C^{14}).*

during starvation were labeled with uracil-C^{14} and recovery from starvation was accomplished in the presence of an excess of uracil-C^{12} and cytosine-C^{12}. The method used to transfer cells from deficient to complete medium also removed the existing radioactive uracil pool (*see* Sypherd, 1965). The pools were then quickly repopulated with nonradioactive molecules, which provided a trap for C^{14} pyrimidines derived from any RNA breakdown. There was essentially no dilution of radioactivity in acid-insoluble material when recovery took place in nonradioactive medium (Fig. 3).

The distribution of relaxed RNA among ribonucleoprotein particles was demonstrated in a previous paper (Sypherd, 1965). The fate of these particles during recovery and subsequent growth was examined by isotopically labeling the RNA formed during starvation, and allowing recovery to occur in the absence of radioactive isotope. The times at which samples were taken for zone centrifugation analyses are shown in Fig. 4A. Figures 4B, C, D, and E show the redistribution of the RNA residing in relaxed particles. It is clear from these analyses that the lighter relaxed particles undergo gradual shifts in size, until after

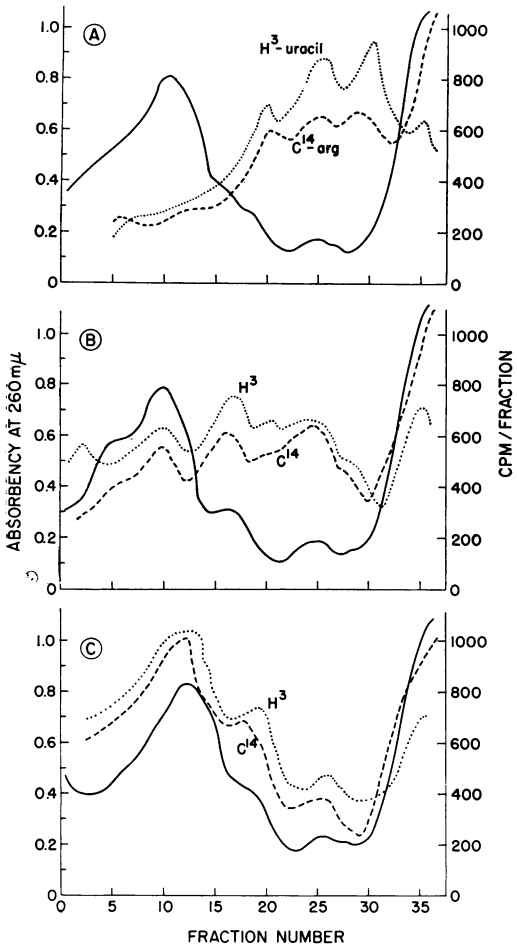

FIG. 5. *Distribution of arginine-C^{14} and uracil-H^3 incorporated into trichloroacetic acid-insoluble material of a histidine-starved culture of HAR. (A) Absorbancy (solid line), arginine-C^{14} (broken line), and uracil-H^3 (dotted line) profiles immediately after termination of starvation. (B) Profiles obtained from cells taken 45 min after termination of starvation. (C) Profiles obtained from cells taken 80 min after termination of starvation.*

about 80 min, the radioactivity profile closely follows the optical density of native 70*S*, 50*S*, and 30*S* ribosomes (Fig. 4F). At intermediate times (i.e., between 25 and 50 min), the particle sizes are quite heterogeneous, representing *S*-values of 70, 50, 45, 30, and 20*S*. It can also be seen, by comparing Fig. 4B with 4C, that essentially no change occurred during the first 15 min after the addition of arginine.

The conversion of relaxed particles to mature ribosomes was also demonstrated by labeling the protein of the particles. The flow of radioactivity from relaxed particles labeled with arginine-C^{14} into mature ribosomes proceeded in the same manner as the RNA. Zone centrifugation analyses taken at representative times after the addition of the deprived amino acid are shown in Fig. 5. These experiments, performed by labeling the relaxed particles with arginine-C^{14} and uracil-H^3, demonstrated that the RNA and protein moieties of the relaxed particles become incorporated into mature ribosomes. The C^{14}-H^3 ratios of intermediate peaks formed during the conversion process indicated that the RNA and protein remained associated during this process.

The previous experiments demonstrated the conversion of relaxed particles to mature ribosomes when starvation is terminated and growth allowed to resume. The simplest interpretation of these findings is that the RNP particles accumulated during starvation are direct precursors to mature ribosomes. Evidence which bears on this inference comes from experiments in which the flow of relaxed material into ribosomes was compared directly with the flow of precursors made under stringent conditions. The experiment was performed by first labeling with uracil-C^{14} the RNA made during relaxed starvation. At the termination of starvation, the uracil-C^{14} was replaced with uracil-H^3 to trace that RNA formed under stringent conditions during the growth lag. The quantity of uracil-H^3 added was exhausted after 15 min. The incorporation of both isotopes is shown in Fig. 6. Samples were taken

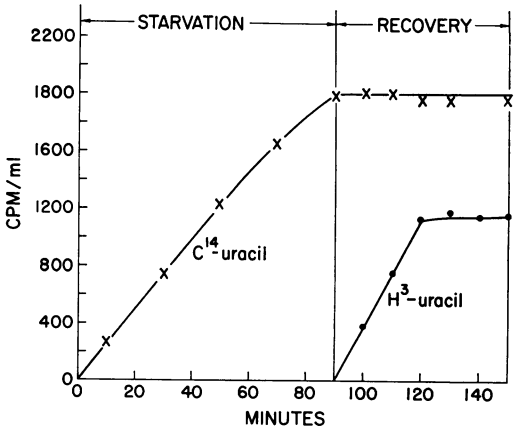

FIG. 6. *Rates of incorporation of uracil-C^{14} during starvation and uracil-H^3 during recovery in a culture of HAR. The termination of uracil-C^{14} incorporation was accomplished as described in Materials and Methods. Uracil-H^3 was added to the culture, along with* L*-histidine, to initiate the recovery phase. The net quantities of RNA formed during the incorporation stages were 72 and 19 μg/ml for the starvation and recovery periods, respectively.*

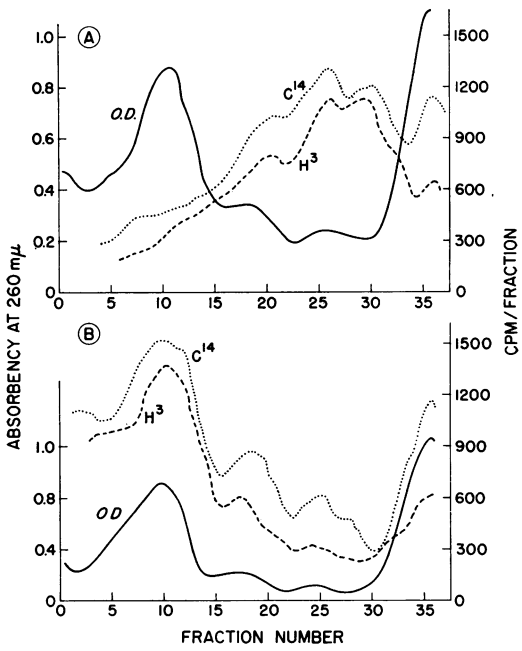

FIG. 7. *Distribution of radioactive pyrimidines incorporated under relaxed and stringent conditions. A culture of HAR was starved for arginine and uracil-C¹⁴ incorporated into nucleic acids. The starvation was terminated by the addition of arginine, and uracil-H³ was used to label nucleic acids formed during the recovery period. (A) Distribution of both isotopes 15 min after the termination of starvation, and immediately after exhaustion of uracil-H³. (B) Redistribution of both isotopes 75 min after the termination of starvation.*

for sucrose-gradient analyses at intervals after the exhaustion of the uracil-H³. The H³ label was incorporated into regions of the gradient occupied by relaxed particles (Fig. 7A). Analyses at subsequent times showed that relaxed RNA and the nucleic acid formed under stringent conditions were converted to 70*S* ribosomes at the same rate. This was judged from the constancy of the ratio of C¹⁴-H³ appearing in the 70*S* region and leaving the region occupied by relaxed particles (Table 2 and Fig. 7B). These data show that the biochemical events involved in the conversion of precursors to ribosomes occurred with an equal probability for both the relaxed RNP particles (labeled with C¹⁴) and the "natural" precursor particles (labeled with H³) formed during stringent growth. Thus, in a metabolic sense, the two particle forms are indistinguishable. The incorporation of uracil-H³ into the same peaks occupied by relaxed particles does not represent a completion of relaxed RNA molecules, since these molecules are already the size of RNA's from complete ribosomes (Sypherd, 1965). Moreover, the newly synthesized "stringent" RNA is also of the 16*S* and 23*S* variety (*unpublished data*).

A second set of experiments bears on the relationship of relaxed particles to the ribosome precursors formed during stringent growth. In an attempt to find whether relaxed particles would act as "trapping pools" for natural precursors, cultures of HAR (Sypherd, 1965) were starved for various lengths of time to vary the amount of relaxed material accumulated. Growth was then initiated with the required amino acid, and a 10-

TABLE 2. *Simultaneous conversion of relaxed particles and recovery RNA to mature ribosomes**

Time during recovery	Ratio of C¹⁴ to H³ in 70*S* and relaxed particles	
	Relaxed particles	70*S* ribosomes
min		
15	1.27	—
30	1.30	—
45	—	1.29
60	—	1.30
90	—	1.25

* A culture was starved for arginine and the relaxed RNA was labeled with uracil-C¹⁴. The starvation was terminated, and the cells were resuspended with arginine and a 15-min supply of uracil-H³. At times after the exhaustion of the uracil-H³, samples were analyzed on sucrose gradients. Radioactivity for both isotopes in the 70*S* and the relaxed-particle regions was used for expressing the ratios.

TABLE 3. *Appearance into mature ribosomal particles of RNA formed during recovery**

Time during recovery	Per cent total radioactivity in mature ribosomes		
	Culture A	Culture B	Culture C
min			
20	65	50	40
30	75	65	55
45	100	80	70
60	100	100	85
90	100	100	100

* Three cultures were deprived of arginine for 45 min (A), 90 min (B), and 180 min (C). A 10-min supply of uracil-C¹⁴ was added with arginine at the termination of starvation. Samples were removed at intervals from each culture, and the radioactivity in relaxed (ribonuclease-sensitive) and mature (ribonuclease-resistant) RNP particles was determined.

min supply of uracil-C^{14} was added simultaneously. After 10 min of incorporation, the bulk of the radioactivity was present in the same peaks occupied by relaxed particles. At later intervals, the radioactivity began to appear in the 70*S* region of the gradient. Therefore, the incorporation of uracil-C^{14} into mature ribosomes could be used as a measure of the rate of conversion of natural precursors to ribosomes. In this case, mature ribosomes are defined as those RNP particles resistant to 2 μg/ml of ribonuclease at 15 C (Sypherd, 1965). The time required for conversion of the natural precursors to ribosomes varied directly with the quantity of relaxed particles (Table 3), which was determined by the length of the starvation period. This situation would obtain if the natural precursors entered a "pool" populated by relaxed particles, and if both particles left the "pool" by a mechanism which did not discriminate between the particle types. And finally, as the pool of relaxed material became larger, a correspondingly greater time was required for all the precursor material to become converted to ribosomes.

DISCUSSION

The primary goal of the study was to assess the physiological significance of RNP particles formed during relaxed starvation. The results presented here clearly demonstrate that these RNP particles are converted to mature ribosomes when the bacteria are allowed to recover from amino acid deprivation. The conversion involves both the nucleic acid and protein of the particles, and takes place without the intermediate steps of degradation and resynthesis. This conclusion is based on the evidence that mononucleotides act as the immediate precursors to ribosomal and soluble RNA's, and that no subunit-polynucleotides are involved in their biosynthesis (*see* Spirin, 1963). In the present investigation, the RNA formed during relaxed starvation was labeled with radioactive uracil. When recovery from starvation occurred in a medium containing an excess of nonradioactive uracil, there was no loss of acid-precipitable radioactivity. In addition, the total radioactivity of the soluble RNA fraction did not change during recovery. Therefore, there is no apparent degradation of relaxed RNA to the purine-pyrimidine level, or to molecular classes that sediment with soluble RNA.

Although the data showed that relaxed particles were converted to ribosomes, there remained the question of whether these particles represent normal precursor stages in ribosome biosynthesis. In the absence of adequate physical-chemical criteria for answering this question, the kinetic behavior of these particles in vivo was compared with that of precursor RNA particles formed under stringent conditions. These experiments showed that "natural" precursors (i.e., the RNA synthesized under stringent conditions during recovery from starvation) passed through the same particle stages as relaxed RNA. In addition, the accumulated relaxed particles acted as effective trapping pools for those RNP particles made during recovery. The data further showed that both relaxed particles and natural precursor particles were converted to ribosomes on the basis of a random selection of both particle types. These observations, together with the fact that relaxed particles have nominal *S*-values similar to those of natural precursor particles (Boezi et al., 1961), make rather compelling the conclusion that relaxed RNP particles represent true precursor stages in the formation of mature ribosomes, and are themselves immature ribosomal particles.

Studies with the relaxed mutants, together with the studies of accumulation of RNP particles during treatment with chloramphenicol (Nomura and Watson, 1959), puromycin (Dagley et al., 1962), and 5-fluorouracil (Boezi et al., 1961), pose an interesting problem in the control of ribosome formation. Despite the severe restriction on protein synthesis in these situations, it would seem that some completed ribosomes should appear during the accumulation period. Yet, at no time during the starvation of a relaxed mutant are there precursor particles sufficiently mature to dimerize and form 70*S* units. This is judged from the fact that uracil-C^{14} radioactivity appearing in the 70*S* region during starvation does not sediment with the 50*S* and 30*S* subunits when the 70*S* particles are dissociated in low Mg^{++}. It would seem, then, that there exists a feedback mechanism, involving ribosomes, to regulate the rate of ribosome formation. This mechanism would function separately from the regulation of RNA synthesis, since the latter system is bypassed mutationally in the case of the relaxed mutant. Certain parameters of ribosomal protein synthesis are presently being studied, in an attempt to learn what, if any, role these events play in the regulation of ribosome formation.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Science Foundation to J. A. DeMoss, and to the author (GB-3163).

The excellent technical assistance of Mary Jane Neibel is acknowledged.

LITERATURE CITED

BOEZI, J. A., E. T. BOLTON, R. J. BRITTEN, D. B. COWIE, B. J. MCCARTHY, J. E. MIDGLEY, AND

R. B. ROBERTS. 1961. Carnegie Institution of Washington Year Book 60, p. 301.

DAGLEY, S., G. TURNOCK, AND D. G. WILD. 1963. The accumulation of ribonucleic acid by a mutant of *Escherichia coli*. Biochem. J. **88**:555–566.

NAKADA, D., I. A. C. ANDERSON, AND B. MAGASANIK. 1964. Fate of the ribosomal RNA produced by a "relaxed" mutant of *Escherichia coli*. J. Mol. Biol. **9**:472–488.

NOMURA, M., AND J. D. WATSON. 1959. Ribonucleoprotein particles within chloramphenicol-inhibited *Escherichia coli*. J. Mol. Biol. **1**:204–217.

SPIRIN, A. S. 1963. Some problems concerning the macromolecular structure of ribonucleic acids. p. 301–345. *In* J. N. Davidson and W. E. Cohn [ed.], Progress in nucleic acid research, vol. 1. Academic Press, Inc., New York.

SYPHERD, P. 1965. Accumulation of ribonucleoprotein particles in a relaxed mutant of *Escherichia coli*. J. Bacteriol. **90**:403–410.