Ribosome Synthesis in *Escherichia coli* Treated with 5-Fluorouracil*

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ABSTRACT: 5-Fluorouracil (FU) interferes with the synthesis of ribosomes in *Escherichia coli* resulting in the accumulation of a number of abnormal nucleoproteins (FU-particles) which can be seen on sucrose-density gradients sedimenting at 27 S, 33 S, and 42 S. These contain the usual 16 S and 23 S ribosomal ribonucleic acids but differ from normal ribosomes in their stability. Fluoropyrimidine can be incorporated into stable, normal-appearing ribosomes when excess uracil is available during treatment with fluorouracil. The possibility that FU-particles are ribosome precursors has been investigated by examining the fate of these components during a recovery period following withdrawal of the analog. Our findings show that the ribonucleic acid (RNA) formed during fluorouracil treatment is unstable and readily breaks down within the cells to acid-soluble materials. Unless ribonucleic acid synthesis is inhibited or uracil is present to compete with the fluoropyrimidine, the analog in the degradation products is reincorporated into RNA. When continued RNA synthesis is inhibited with actinomycin D, in cells made sensitive to the antibiotic with EDTA, there is no conversion of FU-particles into normal ribosomes during the recovery period. FU-particles are, therefore, not intermediates in ribosome biosynthesis and their apparent transformation to ribosomes under conditions permitting nucleic acid synthesis is the result of a reutilization of RNA breakdown products.

It has been suggested (McCarthy *et al.*, 1962; Kono and Osawa, 1964), largely on the basis of pulse-labeling experiments, that a number of incomplete nucleoprotein particles act as precursors in the biosynthesis of bacterial ribosomes. Particles which may be related to these intermediates are known to accumulate under a variety of circumstances, each of which results in a preferential synthesis of RNA[1] with little or no accompanying synthesis of protein. These conditions include inhibition by the antibiotics chloramphenicol (Nomura and Watson, 1959; Kurland *et al.*, 1962), puromycin (Dagley *et al.*, 1962; Sells, 1964), streptomycin (Dubin, 1964), and chlortetracycline (Holmes and Wild, 1965). Similar components are formed by a strain of *Escherichia coli* as the result of K^+ depletion (Ennis and Lubin, 1965) and by certain amino acid auxotrophs (relaxed mutants) during amino acid starvation (Dagley *et al.*, 1963). These nucleoprotein particles sediment more slowly than mature ribosomes, generally between 18 S and 25 S, are deficient in protein and are especially susceptible to degradation by nucleases. They contain two species of ribonucleic acids corresponding to the usual ribosomal RNA except that the slower moving component sediments slightly faster than 16 S RNA (Kono *et al.*, 1964; Holmes and Wild, 1965).

Recently Kono and Osawa (1964) observed similar components in *E. coli* treated with 5-fluorouracil (FU). Previous experiments (Horowitz and Chargaff, 1959; Horowitz *et al.*, 1958, 1960) had shown that this pyrimidine analog is extensively incorporated into bacterial RNA in place of uracil and has profound effects on protein and nucleic acid synthesis. It was also noted (Aronson, 1961) that FU interferes with ribosome metabolism; Gros *et al.* (1962) concluded that the formation of the 50 S ribosomal subunit, but not that of the 30 S, was inhibited. The more recent evidence of Kono and Osawa (1964), as well as that of Nakada (1965), has indicated, however, that neither the 50 S nor 30 S ribosome is formed, but that abnormal ribonucleoprotein particles (FU-particles), sedimenting in the 30 S region, accumulate in *E. coli* grown in FU. These FU-particles appeared to behave like precursors of normal ribosomes and were thought to represent intermediate steps in the sequence leading to the formation of complete ribosomal particles (Kono and Osawa, 1964).

In a continuation of previous studies with FU we have also observed these abnormal ribosomal components. This report documents our findings which extend the observations previously made and have led us to conclusions markedly different from those described

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[1] Abbreviations used in this work: FU, 5-fluorouracil; FU-particles, 5-fluorouracil-containing ribonucleoprotein particles; DNAase, deoxyribonuclease; RNAase, ribonuclease; SDS, sodium dodecyl sulfate.

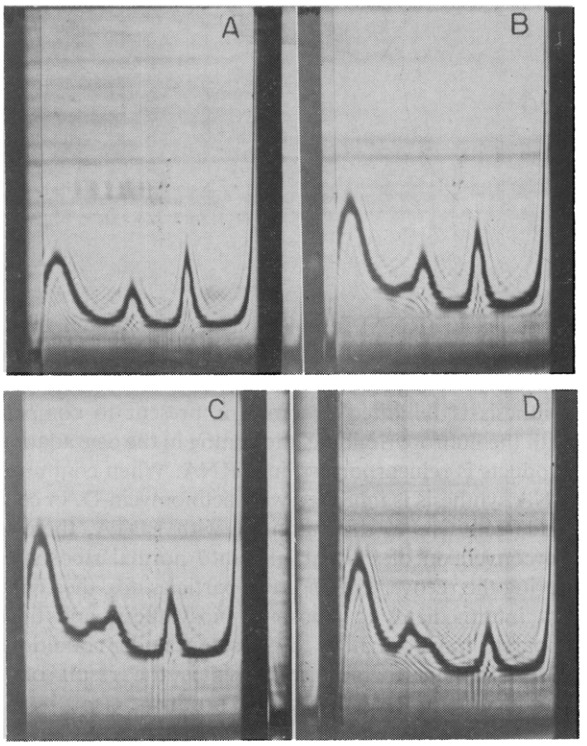

FIGURE 1: Effect of FU on *E. coli* ribosomes. Exponentially growing cultures of *E. coli* B-RA were incubated with 15 μg/ml FU for 0 min (a), 30 min (b), 60 min (c), and 120 min (d). Extracts were prepared in 10^{-3} M Tris–HCl buffer (pH 7.4) containing 10^{-4} M magnesium acetate and 10 μg/ml DNAase. These were examined in the Spinco Model E ultracentrifuge at 31,410 rpm at room temperature. Sedimentation is to the right: (a) normal ribosomes, $s_{20,w}$ 41, 24, and 5; (b) $s_{20,w}$ 42, 27, 21, and 5; (c) $s_{20,w}$ 43, 27, 21, and 4; (d) $s_{20,w}$ 42, 27, 20, and 5.

above. The nucleoprotein particles which accumulate as the result of FU incorporation sediment more slowly than mature ribosomes, are more unstable *in vivo* and *in vitro*, and in general have many of the properties usually ascribed to ribosome precursor particles. However, they are not directly converted to ribosomes following removal of the fluoropyrimidine from the growth medium and do not appear to be precursors of normal ribonucleoprotein components. Andoh and Chargaff (1965), in a recent paper, have reached some of the same conclusions regarding the physiological significance of FU-particles.

Methods and Materials

Bacterial Growth and Media. The majority of experiments were carried out with *E. coli* strain B-RA, kindly supplied by Dr. Rakoma Wiesner. The cells were grown in a glucose–salts medium (Demerec and Cahn, 1953) with aeration by shaking and bacterial growth followed by measuring the turbidity at 650 mμ with a Bausch and Lomb Spectronic 20. 5-Fluorouracil and any other additions, as indicated in the individual experiments, were made when the cells were in the exponential phase of growth. Thymidine was usually added to cultures treated with 5-fluorouracil; however, the results observed were the same whether thymidine was present or not. Those experiments requiring the use of actinomycin D were performed with the strain AB 1105 obtained from Dr. Lotte Leive. These cells were grown in the medium of Levinthal *et al.* (1962) as modified by Leive (1965a). Treatment with EDTA to make the cells permeable to the antibiotic was carried out for 3 min exactly as described by Leive (1965a).

Sucrose-Density Gradients. Bacterial cultures were harvested in the cold by centrifugation, washed twice with 0.001 M Tris–HCl buffer (pH 7.4) containing 10^{-4} M magnesium acetate (standard buffer), and resuspended in 5 ml of this buffer containing 10 μg/ml of DNAase. The cells were broken in a French pressure cell and the extracts clarified by centrifugation at 25,000*g* for 30 min.

Samples of this extract were layered on 24 ml of 5–20% linear sucrose-density gradients (Britten and Roberts, 1960) prepared in standard buffer. Centrifugations were carried out in the SW 25.1 rotor of the Spinco Model L preparative ultracentrifuge at 22,000–25,000 rpm for 6–8 hr at a temperature of *ca.* 4°. Twelve-drop fractions were collected for absorbancy measurement and radioactivity assay. Ten drops, the first and last five of each fraction, were used for determination of radioactivity; the middle two drops were collected separately, diluted with 2.5 ml of standard buffer, and used to measure the absorbancy at 260 mμ in a Beckman DU spectrophotometer. Samples were prepared for counting by precipitation with 7% trichloroacetic acid after the addition of 0.6 mg of bovine serum albumin. The precipitates were washed, dissolved in 1 N ammonium hydroxide, plated, and counted as described below. Approximate sedimentation coefficients were estimated by the method of Martin and Ames (1961).

RNA samples were analyzed on sucrose gradients prepared in a buffer containing 0.01 M sodium acetate (pH 5.1), 10^{-4} M $MgCl_2$, and 5×10^{-2} M NaCl (acetate buffer), and the centrifugation was carried out for 15 hr at 22,500 rpm in the cold.

Preparation of RNA. RNA was prepared from bacterial extracts by a modification of the method described by Kurland (1960). Enough bentonite (Fraenkel-Conrat *et al.*, 1961) and sodium dodecyl sulfate (SDS) were added to the extracts to give a final concentration of 1 mg/ml and 0.5%, respectively. After shaking at room temperature for 1 min, an equal volume of 90% phenol was added to the suspension and shaking continued for 30 min in the cold. The mixture was then centrifuged at 10,000*g* for 5 min and the aqueous layer removed and again treated with phenol. Potassium acetate was added to the resulting aqueous layer to give a final concentration of 0.2 M and the RNA precipitated by the addition of two volumes of ethanol. The precipitate was dis-

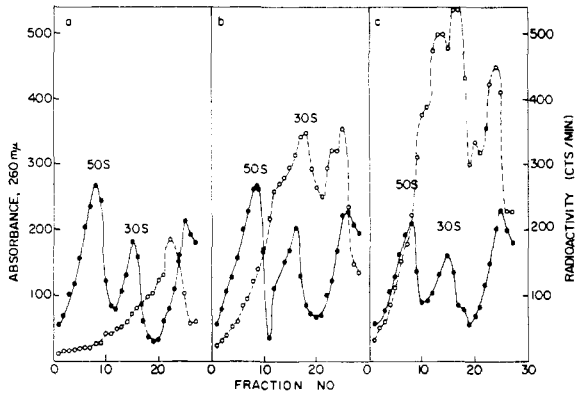

FIGURE 2: Sucrose-density gradient analysis of extracts from FU-treated *E. coli*. Cultures were treated with [2-^{14}C]5-fluorouracil (15 μg/ml) for 3 min (a), 30 min (b), and 60 min (c). The specific activity of the fluoropyrimidine was 0.67 μc/mg in (a) and 0.33 μc/mg in (b) and (c). At the times indicated 0.01 M NaN_3 was added to the cultures and extracts prepared as for Figure 1. Centrifugation was for 7 hr at 25,000 rpm in a sucrose gradient containing 10^{-3} M Tris-HCl (pH 7.4) and 10^{-4} M magnesium acetate. Radioactivity (O- - - -O) and absorbance at 260 mμ (●——●) were measured as described in the text.

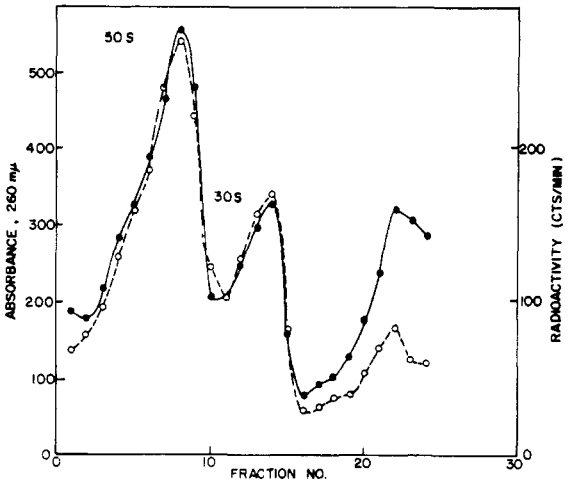

FIGURE 3: Incorporation of FU in the presence of exogenous uracil. An exponentially growing culture of *E. coli* B-RA was incubated for 60 min with [2-^{14}C]5-fluorouracil (0.005 μc = 15 μg/ml) and 20 μg/ml uracil. Further metabolism was stopped by the addition of 0.01 M NaN_3 and extracts were prepared as usual (Figure 1). Centrifugation, 25,000 rpm for 7 hr in a sucrose gradient containing 10^{-3} M Tris-HCl (pH 7.4) and 10^{-4} M magnesium acetate; (●——●), absorbance at 260 mμ; (O- - - -O), radioactivity.

solved in acetate buffer and any insoluble residue removed by centrifugation.

Analytical Methods. Chemical analyses and determination of the extent of radioisotope incorporation into cells were carried out on samples precipitated with 7% trichloroacetic acid, washed once with the same solution, and dissolved in 1 N ammonium hydroxide. Radioactivity was determined by evaporating aliquots of the sample to dryness on planchets which were then counted in a Nuclear-Chicago D47 gas-flow counter equipped with micromil window. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The orcinol method (Mejbaum, 1939) was used to determine RNA, with *E. coli* RNA serving as a standard.

Analytical ultracentrifugations were performed at room temperature in a Spinco Model E ultracentrifuge equipped with schlieren optics, at a rotor speed of 31,410 rpm. The sedimentation coefficients reported were corrected to water at 20° but not for concentration effects.

Materials. 5-Fluorouracil was a gift from Dr. W. E. Scott of Hoffmann-LaRoche, Inc. Dr. A. Stone of Merck Sharp and Dohme generously provided the actinomycin D. All carbon-labeled compounds were purchased from the California Corp. for Biochemical Research. Crystalline ribonuclease and deoxyribonuclease were obtained from Worthington Biochemical Corp.

Results

Effect of 5-Fluorouracil on Ribosome Formation. Bacterial cells exposed to FU synthesized ribonucleoprotein particles which could be observed in the analytical ultracentrifuge as new components sedimenting somewhat slower than the 30 S ribosomal subunit (Figure 1). The extracts examined were prepared in standard buffer containing 10^{-4} M magnesium acetate. Under these conditions controls not treated with FU showed 50 S and 30 S ribosomes as well as the slower 4 S peak produced by the soluble-cell components (Figure 1a). Following a 30-min treatment with the fluoropyrimidine a new boundry sedimenting at 20–22 S (uncorrected for concentration effects) began to appear (Figure 1b). More of this component was evident at later times and after 2 hr of exposure to FU it represented the bulk of the material in the 30 S region (Figure 1c, d).

The events occurring during the incubation with FU were studied in greater detail by labeling the RNA formed with [2-^{14}C]5-fluorouracil and examining extracts on sucrose-density gradients (Figure 2). After a short, 3-min, exposure to the analog, most of the label was found to sediment between the soluble fraction and the 30 S ribosomes in the region expected for m-RNA (Figure 2a). Extracts prepared after 30 min and 60 min (Figure 2b and c) showed a number of FU-containing components: two sedimented in the 30 S region, one slightly faster, 33 S, the other somewhat slower, 27 S, than normal 30 S ribosomes (*cf.* Kono and Osawa, 1964); another FU-particle sedimenting at about 42 S was also observed. Little or no FU was found in the region occupied by the 50 S ribosomal subunit. The soluble fraction of the cell, seen at the top of

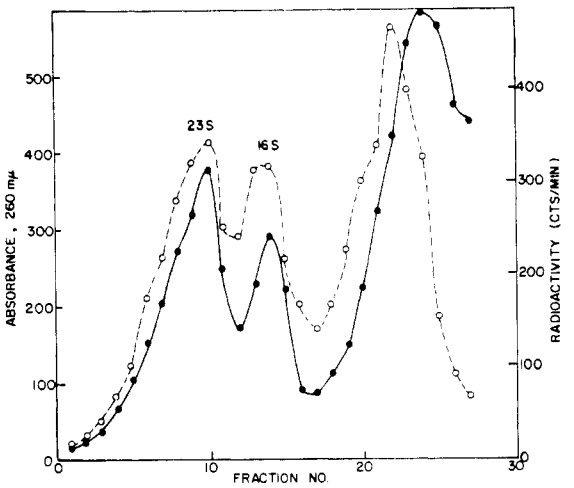

FIGURE 4: Sedimentation analysis of RNA from *E. coli* grown in 5-fluorouracil. [2-^{14}C]5-Fluorouracil (0.005 μc = 15 μg/ml) and thymidine (25 μg/ml) were added to cultures of *E. coli* B-RA. After 30 min the cells were harvested and the RNA prepared by the phenol–SDS method. Centrifugation, 22,500 rpm for 15 hr in a sucrose gradient containing 0.01 M sodium acetate (pH 5.1), 10^{-4} M $MgCl_2$, and 5×10^{-2} M NaCl; (●——●), absorbance at 260 mμ; (O- - - -O), radioactivity.

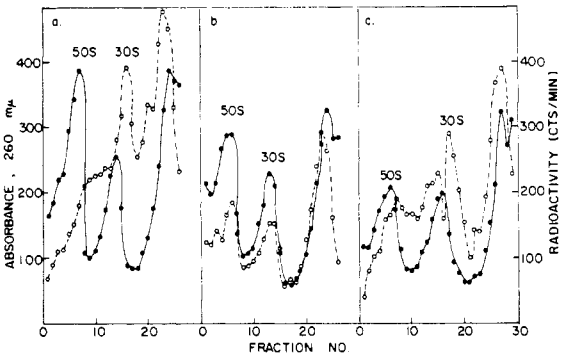

FIGURE 5: Recovery of *E. coli* from 5-fluorouracil treatment in the presence and absence of uracil. After 30 min of exposure to [2-^{14}C]5-fluorouracil (0.005 μc = 15 μg/ml) and thymidine (25 μg/ml) the cells (*E. coli* B-RA) were washed free of the fluoropyrimidine, resuspended in growth medium, and the culture divided into three portions. One was harvested immediately without further incubation (a). Uracil (20 μg/ml) was added to the second (b); no further additions were made to the third (c), and (b) and (c) were then incubated for 20 min. The cell extracts and sucrose gradients contained 10^{-3} M Tris–HCl (pH 7.4) and 10^{-4} magnesium acetate; centrifugation, 22,500 rpm for 8 hr; (●——●), absorbance at 260 mμ; (O- - - -O), radioactivity.

the gradient, also contained large amounts of FU. For reasons as yet unknown, the peak of labeled material generally sedimented slightly faster than the ultraviolet absorption peak.

Preliminary experiments (J. Horowitz, 1965, unpublished observations) had shown that essentially all the labeled FU was incorporated into the RNA of the cells and all of this could be recovered as 5-fluorouridylic acid following alkaline hydrolysis and separation of the nucleotides on Dowex-2.

Influence of Uracil on 5-Fluorouracil Incorporation. The results observed were quite different when uracil was present during the incubation with FU. Under these conditions none of the abnormal FU-particles could be seen in extracts analyzed on sucrose gradients. Figure 3 shows the results of an experiment in which cells were treated with [^{14}C]-labeled FU and uracil for 60 min. Analog was incorporated into components which sedimented like normal ribosomes; the pattern of FU incorporation closely followed the ultraviolet absorption profile. The specific activity of components on the gradient, the counts per minute incorporated per unit absorbance at 260 mμ, was only about one-third that found in cells treated with FU alone. This agreed well with previous experiments which had shown that FU was as extensively incorporated in the presence of exogenous uracil as in its absence and that the amount of RNA formed was approximately three times as great with uracil as without (Horowitz *et al.*, 1960).

Properties of FU-particles. Examination on sucrose gradients of the ribonucleic acids extracted from FU-treated cells showed the fluoropyrimidine present in both the ribosomal RNA species, 23 S and 16 S, as well as in the low-molecular weight RNA fraction, Figure 4 (see also Kono and Osawa, 1964). Thus, although none of the analog was found in the 50 S ribosomes, FU appeared in the 23 S RNA component usually associated with this ribosomal subunit (Kurland, 1960). The 16 S and soluble FU–RNA appeared to sediment slightly ahead of the corresponding ultraviolet absorbing peak.

The FU-particles are quite unstable both *in vivo* (see later) and *in vitro*. It was found that they were highly sensitive to digestion by pancreatic ribonuclease: incubation of extracts, prepared from cells grown in the presence of the fluoropyrimidine, with 1 μg/ml of RNAase at 26° for 15 min resulted in an almost complete loss of the FU-particles. Normal ribosomes, as judged by the ultraviolet absorbancy profile following centrifugation on a sucrose-density gradient, seemed to be considerably more resistant to this treatment as was the FU–RNA in the soluble fraction of the cell.

That the components synthesized during treatment with FU are composed partly of protein was demonstrated by the incorporation of [^{14}C]amino acids into the FU-particles: sucrose-density gradient analysis (not presented) of extracts from cells exposed to unlabeled FU and [1-^{14}C]valine showed that the pattern of amino acid incorporation closely resembled that of FU incorporation.

It is not entirely clear whether the FU-particles can associate reversibly as the magnesium ion concentration is increased. FU-containing components sedimenting

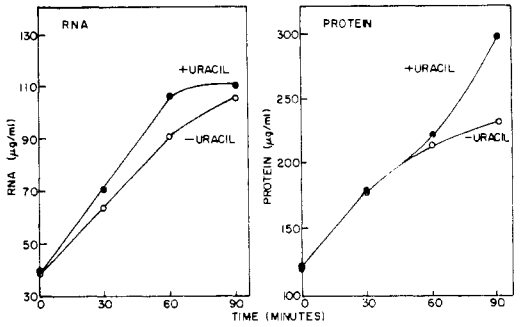

FIGURE 6: Protein and RNA synthesis by FU-treated *E. coli* after removal of FU. 5-Fluorouracil (15 μg/ml) and thymidine (50 μg/ml) were added to cultures of *E. coli* B-RA. After 60 min the cells were harvested, washed three times with growth medium lacking FU, and resuspended in the original volume of this medium. The suspension was divided into two portions, one received no further addition (○), the other 20 μg/ml uracil (●). Both were incubated at 37° and samples removed at intervals for analysis of RNA and protein.

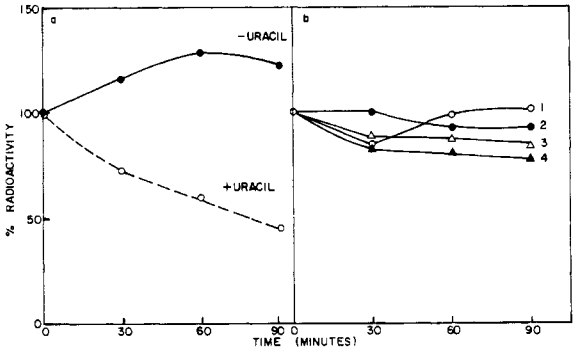

FIGURE 7: Loss of 5-fluorouracil from bacterial cells. (a) *E. coli* B-RA was exposed to [2-^{14}C]5-fluorouracil (0.005 μc = 15 μg/ml) for 30 min; the cells were washed, resuspended in fresh medium, and divided into two portions. No further additions were made to one culture (●); the other received uracil (20 μg/ml) (○) and both were incubated at 37°. Samples were removed at intervals for measurement of the cold trichloroacetic acid precipitable radioactivity. (b) The procedure was essentially that described in (a); *E. coli* B-RA was treated with FU (15 μg/ml), in the presence of thymidine (25 μg/ml) and [8-^{14}C]adenine (0.001 μc = 10 μg/ml), for 30 min. After washing, the cells were resuspended in fresh medium and divided into four portions containing the following: (1) uracil (20 μg/ml), (2) no further addition, (3) uracil (20 μg/ml) as well as adenine and adenosine (50 μg/ml each) plus guanine and guanosine (10 μg/ml each), (4) the same as (3) without the uracil. Samples were removed and analyzed as in (a). The results are expressed as per cent of the value at the start of incubation in 5-fluorouracil-free medium.

faster than 50 S ribosomes could be seen in extracts prepared in 10^{-2} M magnesium acetate. However, these particles appeared to be heterogeneous and the material in them was extremely sensitive to ribonuclease digestion. In general they did not behave like the usual 70 S ribosomes and may have arisen as the result of nonspecific aggregation. When examined in the analytical ultracentrifuge, extracts (10^{-2} M Mg^{2+}) of FU-treated cells exhibited an unusual distribution of ribosomal particles compared to normal cells: relatively fewer 70 S–100 S particles were present and a larger proportion of the material sedimented in the peaks having low S values, 50 S and under.

Recovery from 5-Fluorouracil Treatment. To test whether the FU-containing ribonucleoprotein particles can be converted to ribosomes, their fate after removal of the base analog from the growth medium was studied. When uracil was present during the recovery period the fluoropyrimidine in the FU-particles rapidly appeared in the ribosomes. Figure 5 depicts the results of an experiment in which the bacteria were first treated with [2-^{14}C]5-fluorouracil for 30 min, then washed free of the analog, and resuspended in growth medium containing uracil. Sucrose gradient analysis of the cells after 20 min of recovery showed that the FU-particles accumulated during the incubation with FU (Figure 5a) had disappeared; the labeled FU now sedimented with the 30 S and 50 S ribosomal particles (Figure 5b).

The conversion to ribosomes was much less rapid when uracil was not present during the recovery. This is shown in Figure 5c; after 20 min, FU-particles could still be observed in these cells. Eventually, however, even in the absence of uracil, the FU did shift into the 50 S and 30 S ribosomes. This difference in the rate of conversion with and without uracil was surprising since it could be shown that during the recovery period following exposure to FU, the cells readily synthesized both RNA and protein at rates which were essentially independent of the presence of uracil for at least 60 min (Figure 6).

Loss of 5-Fluorouracil from RNA. The transformation of FU-particles into ribosomes during recovery from FU-treatment suggests that these components may be direct precursors of ribosomes. However, the possibility that the FU-containing RNA is rapidly degraded and the breakdown products reutilized for ribosome synthesis must also be considered. For this reason the metabolic stability of FU-containing RNA during the recovery period was studied.

Closer examination of sucrose-gradient analyses such as those in Figure 5 gave an indication that recovery in the presence of uracil resulted in some loss of FU from the cells: the specific radioactivity of the components in the gradient, the ratio of trichloroacetic acid precipitable counts to absorbance at 260 mμ, decreased during incubation in the presence of exogenous uracil but not in its absence (*cf.* Figure 5b and c). This loss of FU was examined in greater detail. Cells which had been exposed to [^{14}C]-labeled FU for 30 min were washed free of the fluoropyrimidine and the fate of the incorporated

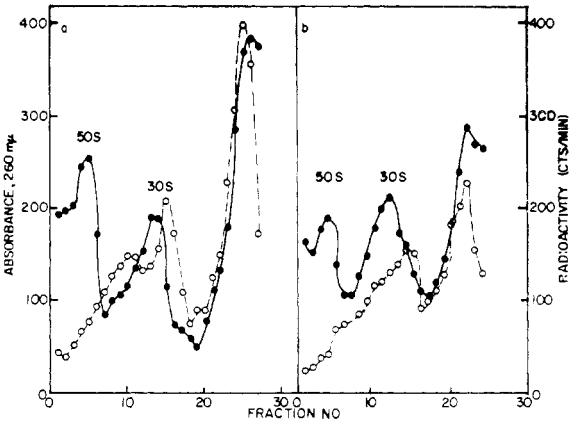

FIGURE 8: Fate of FU-particles in the presence of 5-fluorouracil and uracil. *E. coli* B-RA was exposed to [2-^{14}C]5-fluorouracil (0.005 μc = 15 μg/ml) in the presence of thymidine (25 μg/ml) for 30 min. The cells were washed free of the radioactive analog, resuspended in medium containing unlabeled FU and thymidine at the same concentration used above, and again incubated at 37°. After 15 min, half the culture was removed and prepared for analysis (a). Uracil (20 μg/ml) was added to the other portion and incubation continued for 30 min (b). Cell extracts and gradients contained 10^{-3} M Tris–HCl (pH 7.4) and 10^{-4} M magnesium acetate; centrifugations, 22,500 rpm for 8 hr; (●——●), absorbance at 260 mμ; (○- - -○), radioactivity.

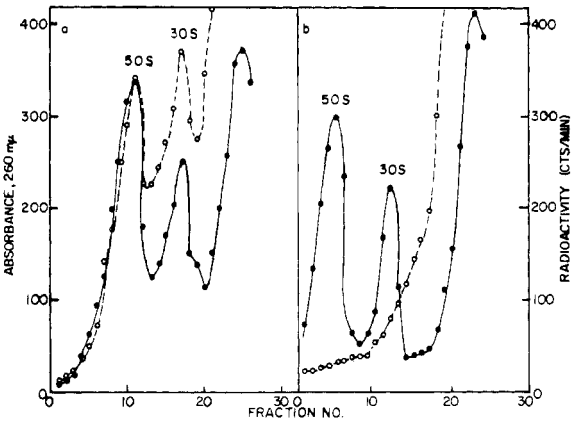

FIGURE 9: Effect of actinomycin D on the conversion of FU-particles to ribosomes. *E. coli* AB 1105 was treated with [2-^{14}C]5-fluorouracil (0.05 μc = 15 μg/ml) and thymidine (25 μg/ml) for 30 min. The cells were washed free of FU, resuspended in 0.12 M Tris–HCl (pH 8.0), and treated with 10^{-3} M EDTA for 3 min to make the cells permeable to actinomycin D. The suspension was then diluted ten times with normal medium containing uracil (20 μg/ml), and the culture divided into two portions: (a) received no further addition while actinomycin (10 μg/ml) was added to the other (b). Both were incubated at 37° for an additional 30 min. Cell extracts and sucrose gradients contained 10^{-3} M Tris–HCl (pH 7.4) and 10^{-4} M magnesium acetate; centrifugations, 22,500 rpm for 8 hr; (●——●), absorbance at 260 mμ; (○- - -○), radioactivity.

label followed during further incubation (recovery) in fresh medium with and without uracil. It was found that the cells lost more than 50% of the FU incorporated within 90 min when uracil was present (Figure 7a). An inspection of sucrose gradients, Figure 5b, indicated that the greatest loss occurred in the ribosomal fraction; the soluble-RNA appeared to be more stable.

In the absence of uracil no label was lost from the acid-insoluble fraction of the cells; in fact, a small increase was usually observed, presumably the result of a continued incorporation of labeled FU from the soluble pools. This stability of FU–RNA in the absence of uracil is deceiving. When synthesis of new RNA was inhibited with actinomycin D, in cells of AB 1105 made sensitive to the antibiotic by treatment with EDTA (Leive, 1965a), more than 70% of the FU incorporated was lost during 90 min of recovery even though no uracil was present. It, therefore, appears likely that FU–RNA is unstable and is readily degraded whether uracil is present or not. The instability of FU-containing RNA was manifest even in the continued presence of FU: cells labeled with [^{14}C]FU and then transferred to medium containing excess cold FU lost more than 35% of the incorporated radioisotope within 15 min.

This instability of ribonucleic acids in FU-treated cells was not as readily apparent when the RNA formed in the presence of the analog was labeled with [^{14}C]-adenine. The labeled purine was incorporated into the regions of the gradient containing the FU-particles, the profile of radioactivity closely resembled that found when [^{14}C]FU was employed (see Figure 2). However, in these experiments relatively little label was lost during the recovery from FU treatment. Even when uracil and excess cold purines and purine nucleosides were present, <20% of the adenine left the acid-insoluble fraction (Figure 7b). These results indicate the difficulties involved in interpreting experiments in which the RNA formed during FU treatment is labeled with nucleic acid precursors other than the fluoropyrimidine itself.

Fluorouracil-containing ribonucleic acids or nucleoproteins are not necessarily unstable. It was shown earlier that the analog incorporated into cells in the presence of uracil is found to sediment with the ribosomes (Figure 3). The RNA formed under these conditions is quite stable; only 1% of the incorporated FU was lost from these cells during 30 min of recovery with uracil and even after 60 min <7% of the label was lost.

FU-Particles as Ribosome Precursors. Two types of experiments were carried out to test whether FU-particles are direct ribosome precursors. The first had its basis in the observation that FU incorporated in the presence of uracil is found in complete ribosomes (Figure 3). If the FU-particles were intermediates in this process, then the addition of uracil at some point during the incubation with the fluoropyrimidine should result in the conversion of the accumulated FU-particles into

mature ribosomes. An experiment designed to check this possibility indicated they were not so converted. *E. coli* B-RA was treated with [^{14}C]FU for 30 min; the cells were then washed free of the labeled fluoropyrimidine and resuspended in fresh medium containing unlabeled analog. After 15 min, to allow for the depletion of radioisotope in the acid-soluble pools, uracil was added and the cells incubated for another 30 min. The results, in Figure 8, show that the FU-containing components were not transformed into ribosomes during the incubation with cold FU and uracil; some labeled FU was lost from the cells during this period due to the lability of the FU-particles.

The conclusions of this experiment were confirmed in a more direct manner by examining the fate of FU-particles during recovery from FU treatment in the presence of actinomycin D to prohibit the synthesis of new RNA. Due to difficulties experienced in making strain B-RA susceptible to actinomycin D, these experiments were carried out with strain AB 1105 which Leive (1965a) found could readily be made sensitive to the antibiotic. 5-Fluorouracil affected this strain in essentially the same way it did *E. coli* B-RA. In the procedure used the cells were allowed to accumulate FU-particles, labeled with [^{14}C]FU, for 30 min. The bacteria were freed of the analog and treated with EDTA for 3 min (Leive, 1965a) to make them permeable to actinomycin. The sensitized cells were then diluted with growth medium containing uracil, divided into two parts, and incubated for 30 min; one portion of the culture contained the antibiotic while the other, serving as control, did not. During the incubation period, the trichloroacetic acid precipitable radioactivity in the cells of both flasks decreased. Almost 70% of the labeled FU was lost from the actinomycin D treated bacteria compared to a 40% loss from the cells not exposed to the antibiotic. Sucrose-gradient analysis of extracts prepared from these cells showed that in the absence of actinomycin, as described earlier for *E. coli* B-RA, the FU associated with the abnormal particles shifted into the ribosomes (Figure 9a; *cf.* Figure 5). However, when the antibiotic was present, none of the FU was found to sediment with ribosomes; the FU-particles had completely disappeared during the incubation period and only the FU in the s-RNA region remained. Thus, when the continued synthesis of RNA is inhibited, the transformation of FU-particles into mature ribosomes does not take place.

Discussion

The nucleoprotein particles synthesized by *E. coli* in the presence of FU sediment more slowly than normal ribosomes and exhibit a greater lability. In general their properties resemble those of the ribosomal precursor particles discussed earlier (see introduction). Apparent support for the suggestion that FU-particles are also intermediates in ribosome biosynthesis comes from studies of the fate of these abnormal constituents during the recovery of cells from FU inhibition. Under these conditions the FU-particles soon disappear and the fluoropyrimidine shifts into components which sediment like the 50 S and 30 S ribosomal subunits (Figure 5). Largely on the basis of this type of evidence Kono and Osawa (1964) concluded that FU-particles were ribosome precursors.

It seems clear, however, that FU-containing RNA especially that in the nucleoproteins is metabolically quite unstable and readily breaks down within the cells to acid-soluble materials (Figures 5 and 7). This instability could be demonstrated directly only when exogenous uracil was present in the incubation medium; in the absence of uracil FU-containing RNA appeared to be stable (Figure 7). However, this was shown to be due to a turnover of RNA: inhibition of continued ribonucleic acid synthesis during the recovery period by means of actinomycin D resulted in a loss of FU from cells even in the absence of uracil. Evidently FU in the degradation products could be reincorporated readily if no uracil was present whereas excess uracil competed with this reutilization. In these experiments it was noted that EDTA-treated cells in contrast to those not exposed to the chelating agent lost labeled FU when incubated in uracil-free medium without actinomycin. Presumably labeled material leaked out of the cells, made generally more permeable by the EDTA (Leive, 1965c), thus depleting the nucleic acid precursor pools.

The fact that the reincorporated FU was found in components sedimenting like ribosomes rather than in FU-particles can be explained by the results in Figure 3 which show that when uracil was available, FU could be incorporated into particles which behaved like normal ribosomes. 5-Fluorouracil so incorporated was stable and little, if any, was lost on incubation in analog-free medium containing uracil. These observations indicate that incorporation of the analog does not necessarily lead to accumulation of abnormal ribosomal components.

More direct tests of whether FU-particles were convertible to ribosomes showed that they were not. This was demonstrated most clearly by the experiments employing actinomycin D to prohibit synthesis of new RNA, thus avoiding the problems raised by the possibility of reincorporating RNA breakdown products. The accumulated abnormal particles were completely degraded within 30 min under these conditions and no transfer of FU into ribosomes occurred (Figure 9). It was found that actinomycin does not prevent utilization of ribosomal precursor particles: chloramphenicol particles were converted into ribosomes in the presence of actinomycin D (J. Horowitz and D. C. Hills, 1965, in preparation). Since, in addition, there is no evidence that actinomycin makes normally stable RNA labile (Levinthal *et al.*, 1963; Leive, 1965b), these results strongly suggest that FU-particles are not ribosome precursors. A similar conclusion could be drawn from an experiment not involving use of the antibiotic (Figure 8). This indicated that accumulated FU-particles were not converted to ribosomes after addition of uracil midway through the period of incubation with FU. On the basis of the results in Figure 3, such a conversion would

have been expected if FU-particles were intermediates.

The reasons for the accumulation of abnormal nucleoprotein components in FU-treated cells is not yet clear. Perhaps FU interferes with ribosomal protein formation and thus leads to a synthesis of incomplete nucleoprotein particles resembling other abnormal particles known to be missing protein (see introduction). However, many of the observed properties of FU-particles could be due to alterations in the secondary structure of the RNA resulting from the replacement of uracil by FU. Changes in the physical properties of polynucleotides brought about by FU have been noted in the case of transfer RNA (Sueoka and Yamane, 1963), ribosomal RNA (Andoh and Chargaff, 1965; Kono *et al.*, 1964), the RNA of bacteriophage (Shimura *et al.*, 1965), and synthetic polynucleotides containing the analog (Szer and Shugar, 1963). Such structural changes may prevent formation of mature ribosomes by interfering with the addition of protein to incomplete particles or, if the FU-particles already contain a complete complement of protein, they may interfere with the folding of the nucleoprotein into its normal compact structure.

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