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EFFECTS OF FLUOROURACIL ON RNA SYNTHESIS IN *BACILLUS CEREUS*

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Abstract—5-Fluorouracil (FU) decreased the growth rate of *Bacillus cereus*, produced inhibition of synthesis of rRNA and ribosomes, and altered the physical properties of ribosomes. These effects were dependent on FU concentration and independent of retarded growth. The quantity of 23S RNA in the FU-treated cells was diminished to a greater extent than was that of 16S RNA, apparently because of ribonuclease degradation. Most of the RNA synthesized in the presence of FU was in the soluble fraction, and [^{14}C] FU was selectively concentrated in this fraction. Ribosomes containing FU possessed a slightly lower thermal denaturation temperature. The 50S and 30S materials containing FU were unstable in 0·1 mM Mg^{2+}. The ratio of RNA to protein synthesis was decreased in the 70S ribosomal fraction and increased in the soluble fraction of cells inhibited by FU.

THREE mechanisms of action have been proposed to explain the inhibitory effects of 5-fluorouracil (FU) on mammalian, bacterial or viral growth.[1] They are: (1) the inhibition of thymidylate synthetase resulting in inhibition of DNA synthesis; (2) the substitution of FU for uracil in RNA synthesis leading to the production of abnormal RNA species; (3) the utilization of FU nucleotides instead of uracil nucleotides resulting in reduced rate of RNA or DNA synthesis or interference with the function of nucleotide sugar derivatives. In this paper we will confine our discussion to the effects of FU on RNA synthesis.

Various investigators have examined effects of FU in *E. coli* at the subcellular level, as described in a recent review.[2] In general, when cells were grown in the presence of FU and subsequently subjected to density gradient centrifugation, the elution profiles of their ribosomes or ribosomal subunits differed appreciably from normal.[3–5] Ribosomal fractions obtained from FU-treated cultures differed from normal ribosomes in having less sedimentation homogeneity as well as a predominance of slower sedimenting particles. These ribosomes were also characterized by an increased sensitivity to ribonuclease and an abnormal RNA distribution pattern upon sucrose density gradient sedimentation when the Mg^{2+} concentration was lowered from 10 mM to 0·1 mM.[2]

A previous report from this laboratory described the marked inhibition of growth of *Bacillus cereus* grown in the presence of the uracil analog, FU.[6] A selective inhibition of DNA synthesis was observed, whereas protein and total RNA synthesis were not specifically inhibited, and proceeded at the rate of turbidimetric increase of the cells.[7] In addition, it was observed that increasing the concentration of FU above 16 μM did not further depress DNA synthesis although the doubling time of the culture continued to increase.[8]

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The present investigation was undertaken to study the effect of FU on RNA synthesis in *B. cereus* at three subcellular levels: (1) the effect of FU on ribosome synthesis and stability; (2) the effect of FU on RNA synthesis; (3) the effect of FU on the interaction between protein and FU-containing RNA.

MATERIALS AND METHODS

Cultures of *B. cereus* 569 were incubated in a Gyrotory shaker at 37° in a medium containing 26 mM KH_2PO_4, 2 mMMg SO_4, 3 μM $MnSO_4$, 13 μM Fe $(NH_4)_2SO_4$, 8·6 mM K_2SO_4, 16 mM tris, and 10 g of casamino acids per liter adjusted to pH 7·1 with HCl. Growth was monitored turbidimetrically at 540 nm using a Beckman DU spectrophotometer. Cultures were monitored to determine that logarithmic growth rate had been obtained, and additions were made to these cultures at a turbidimetric reading (A_{540}) of 0·1. These additions had no effect on the pH of the bacterial medium.

Membrane filtration technique

Incorporation of radioisotopes was measured by the membrane filtration technique.[9] Two 2-ml aliquots were removed from bacterial cultures at frequent intervals. In order to measure total nucleic acid labeling, one aliquot was added to 2 ml of 10 % TCA and allowed to precipitate at room temperature. The second aliquot was added to 2 ml of 1 N KOH, and incubated at 37° to degrade RNA without degrading DNA. The samples were then collected on membrane filters (B-6 Bac-T-Flex, Schleicher and Schuell Co., Keene, N. H.) and washed with three 2-ml portions of 1 % TCA. The filters were placed in liquid scintillation counting vials and 10 ml Bray's solution[10] was added. Incorporation into RNA was calculated from the difference in radioactivity between the TCA and KOH samples.

Radioisotope counting techniques

All samples were dissolved in 10 ml Bray's solution[10] and counted in a Beckman CPM-100 liquid scintillation counter. Each sample was counted for 10 min or 10,000 CPM. For single isotope counting, the counting efficiency for ^3H, ^{14}C and ^{32}P was 30, 80 and 80 per cent, respectively. When samples containing two isotopes were counted, the samples were counted in a window set to exclude counts due to the lower energy emitter, and an additional window was used that included the lower energy emitter. The counts in this latter window were corrected for counts due to the higher energy emitter.

Prelabeling of bacterial cultures

Control cultures were labeled for 30 min with [8-^{14}C]guanine (20 nc/1·6 μg/ml of culture medium) or [8-^{3}H]guanosine (100 nc/5·3 ng/ml of culture medium) for 30 min. Cells were collected by centrifugation at 10,000 *g* for 10 min in a Sorvall SS-1 centrifuge in a cold-room maintained at 4°. The cells were washed once with fresh medium and resuspended in prewarmed medium to give an A_{540} of 0·08. The cultures were grown to an A_{540} of 0·1, and the experiment was begun.

Pulse-labeling of bacterial cultures

Logarithmically growing cultures of *B. cereus* ($A_{540} = 0{\cdot}1$) were pulse-labeled by the addition of [8-^{3}H]guanosine (100 nc/5·3 ng/ml culture medium). The validity

of the pulse was established by monitoring the incorporation of ^3H into RNA by the membrane filtration technique. The isotope incorporated reached a maximum in less than 3 min, and total radioactivity remained constant for at least one doubling of turbidity in control and FU-treated cultures.

Preparation of bacterial extract

Bacterial cells were centrifuged at 10,000 *g* for 10 min, as described above, and resuspended in 10 mM tris buffer pH 7·8 containing 10 mM magnesium acetate and 60 mM KCl. The suspension was disintegrated ultrasonically for 5 min at 0° in an MSE Model 60 W ultrasonic disintegrator, followed by centrifugation for 30 min at 10,000 *g*. The extracts were dialyzed at 4° for 18 hr, against 500 vol. of 10 mM tris buffer pH 7·8 containing 60 mM KCl and 10 mM or 0·1 mM magnesium acetate before density gradient analysis. The ribosomes from extracts of control and FU-treated cells were isolated by centrifugation in a Spinco Model L ultracentrifuge at 105,000 *g* for 90 min. RNA was prepared according to the method of Grünberger and Mandel.[11]

Density gradient analysis

Samples of 0·2–0·3 ml of extract were placed on top of 5 ml of a linear sucrose gradient (pH 7·8, 10 mM or 0·1 mM Mg^{2+}, 10 mM tris, 60 mM KCl). The samples were centrifuged in a Spinco SW 39 rotor at 39,000 rev/min. Density gradient samples were analyzed using an ISCO Automatic Density Gradient Fractionator (Instrumentation Specialties Co., Inc., Lincoln, Neb.) equipped with an ultraviolet analyzer. Fractions (four drops equal to about 0·16 ml) were collected directly into counting vials.

Ribonuclease assay

The assay of Kalnitsky *et al.*[12] was used with minor modifications. Yeast RNA, RNase and extracts of *B. cereus* were prepared in buffer containing 10 mM tris, 60 mM potassium chloride and 10 mM magnesium acetate adjusted to pH 7·8. The reaction mixture contained 3 mg RNA, RNase, or cellular extract and buffer to a final volume of 3·0 ml. Samples were incubated for 30 min in a 37° water bath with continuous agitation. The reaction was terminated by the addition of 2·0 ml of 0·75 % uranyl acetate (w/v) in 25 % perchloric acid (w/v). Samples were placed in an ice-water bath and allowed to cool for 60 min and then were cleared by centrifugation. The supernatant of all samples was diluted 10-fold and the absorbancy was determined at 260 nm against a blank that contained no source of RNase (no extract or RNase) in a Beckman DU spectrophotometer. The results are expressed as units of RNase where one unit of RNase activity is equivalent to that amount of acid-soluble oligonucleotide which causes an increase of absorbancy at 260 nm of 1·0/min. In order to express specific activity, units/mg protein, protein was determined by the method of Lowry *et al.*[13]

Melting curves

Thermal denaturation profiles of ribosomes were obtained with a Beckman DU spectrophotometer attached to a Gilford (model 2000) multirange absorbance recorder. Ribosomes from control and FU-treated cells were tested at initial A_{260} values of 0·7–1·0.

Materials

[2-^{14}C]FU was purchased from Calbiochem, Bethesda, Md. [8-^{14}C]Guanine, ^{14}C-leucine, [8-^{3}H]guanosine and [^{32}P]-phosphate were obtained from New England Nuclear Corp., Boston, Mass. Yeast RNA and RNase A were purchased from Worthington Biochemical Corp., Freehold, N.J. Sucrose for density gradients was RNase-free and obtained from Schwartz Bioresearch, Inc., Orangeburg, N.Y. All other chemicals were purchased from Fisher Scientific, Philadelphia, Penn. FU (NSC-19893) was kindly donated by the Cancer Chemotherapy National Service Center, Bethesda, Md.

RESULTS

(1) *Effect of FU on ribosome synthesis and stability*

Synthesis of ribosomes in the presence of FU. Previous reports from this laboratory have shown that FU inhibited the growth of *Bacillus cereus* and increased the doubling time as measured by turbidity at 540 nm.[7] Control and inhibited cultures grown at 37° to identical turbidities incorporated essentially the same amount of [8-^{14}C]guanine into the total RNA of the cells, establishing that the total synthesis of RNA was equal in both cases.[7] However, the RNA of FU-treated cultures was atypical in that the RNA contained FU instead of uracil.[7]

To determine if FU incorporation altered the synthesis of specific subcellular components, extracts from control and 48 μM FU-treated cultures were subjected to sucrose density gradient analysis. The results are seen in Fig. 1. The distribution patterns of the control extract (Fig. 1a) were characterized by a large peak at tube 8, corresponding to 70S ribosomes, and smaller peaks at tubes 13 and 19, corresponding to the 50S and 30S ribosomal subunits, respectively. The soluble fraction (4S) seen in tube 26 was smaller than the 70S ribosomal fraction. The distribution of radioactivity parallels the absorbancy in control extracts. In contrast, the absorbancy pattern and radioactivity distribution are quite different in extracts from FU-treated cultures (Fig. 1b). The incorporation of guanine into the 70S ribosome was depressed to the point that it appears as a shoulder of the 50S material. The 50S and 30S material were both increased in relation to the 70S material. Isotopic incorporation into the 4S fraction was markedly increased. The absorbancy pattern suggests a depression of synthesis of the 70S ribosome with a concomitant increase in the 50S, 30S and 4S regions. The greatest increase was seen in the 4S fraction.

In order to quantitate alterations in relative distribution of radioactivity in the ribosomal and soluble areas of the gradient, we defined the ribosomal region as tubes 1–19 and the soluble region as tubes 21–28. The results were expressed as percentage radioactivity in the ribosomal fraction. When the gradients in Fig. 1 were analyzed in this manner, a quantitative reduction in incorporation of isotope into the ribosomal fraction was observed. The per cent radioactivity found as ribosomal in the control extract was 64·3 whereas only 32·3 was found as ribosomal in extract from FU-treated culture.

Stability of prelabeled ribosomes during growth inhibition by FU. The virtual absence of 70S ribosomes and the presence of large quantities of material sedimenting about 50S and 30S in FU-treated cultures, suggested that ribosomes synthesized may be broken down to ribosomal subunits during growth in the presence of FU.

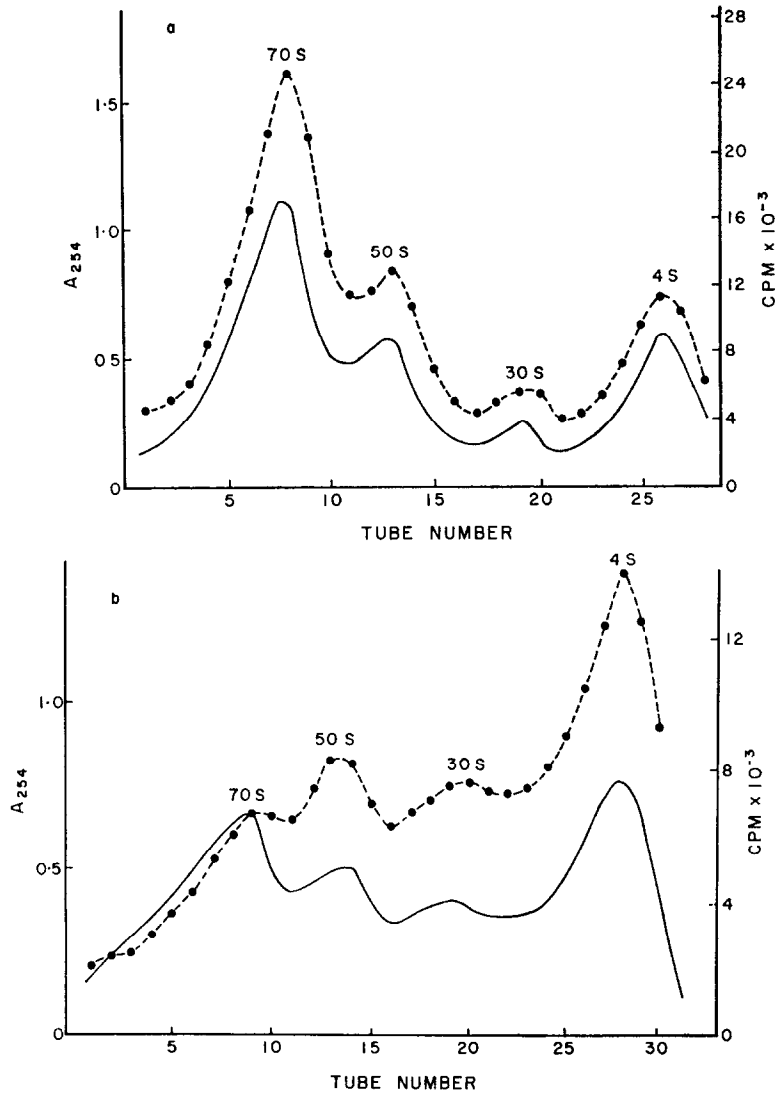

FIG. 1. Distribution of absorbance and radioactivity following sucrose gradient analysis of *B. cereus* extracts from control and FU-treated cells. Parallel cultures were incubated with and without 48 μM FU, and [8-^{14}C]guanine (20 mμc/3·3 μg/ml) was added to each flask. After one doubling of turbidity cells were harvested and extracts prepared in 10 mM Mg^{2+} buffer. These extracts were layered over 5–20 per cent sucrose and centrifuged for 2 hr at 39,000 rev/min in a Spinco SW 39 rotor. Fractions were collected as described in Methods. (a) Control, (b) 48 μM FU: Absorbancy at 254 nm, ———; CPM, – – – –.

To determine the stability of normal ribosomes during growth in the presence of FU, a culture of *B. cereus* was prelabeled with [^{14}C]guanine as described in Methods. This culture was resuspended, divided and grown in the presence and absence of 48 μM FU for one doubling of turbidity (120 and 50 min, respectively). No evidence of instability was noted, as evidenced by the constancy of radioactivity following membrane filtration of cells sampled during the second growth phase, confirming

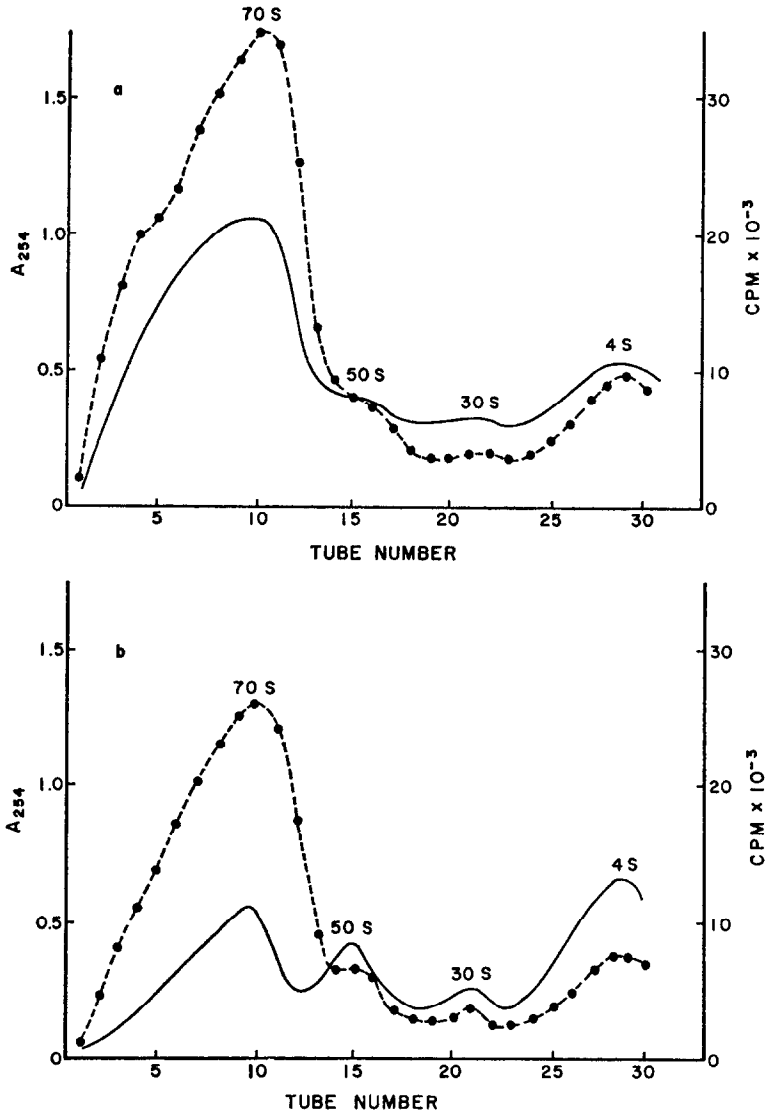

FIG. 2. Stability of preformed ribosomes. A control culture was labeled with [8-^{14}C]guanine, harvested and washed free of guanine. The cells were resuspended in fresh medium. One-half served as control (a) and to other half FU was added to a concentration of 48 μM (b). After one doubling of turbidity, extracts were prepared in 10 mM Mg^{2+}. Extracts were layered over 5–20 per cent sucrose gradients and centrifuged for 110 min as described in Methods. Absorbancy at 254 nm, ———; CPM, – – – –.

previous experiments.[7] Both cultures were then harvested at the end of one doubling of turbidity and analyzed by the density gradient technique (Fig. 2). Characteristic 254 nm absorbance and radioactivity profiles for control cells were observed, showing mainly the 70S ribosomes and a relatively small 4S component (Fig. 2a). The decreased ribosomal content relative to the 4S component typical of FU-treated cultures is shown in the absorbance profile of Fig. 2b. However, the distribution of radioactivity

between the ribosomal and soluble fractions was unaltered by subsequent growth in the presence of FU (control, 65·2 per cent ribosomal; FU-treated 65·0 per cent ribosomal). This demonstrates that little or no breakdown and reutilization of the pre-existing ribosomes had taken place in the presence of FU.

Effect of endogenous and exogenous RNase on ribosomes. In order to test whether an increase in RNase activity due to FU treatment could account for some of the observations reported here, the effect of RNase on ribosome stability was investigated. Extracts were prepared from control and FU-treated (48 μM) cultures after one doubling of turbidity, and were each divided into three fractions. Fraction 1 was used as a control and was not incubated, but was stored in the cold. Fraction 2 was used as a measure of the effect of endogenous RNase on ribosome stability. It was found that extracts from control and FU-treated cells contained 0·16 and 0·20 units

TABLE 1. DEGRADATION OF RIBOSOMES BY RNASE

	Per cent degradation of ribosomes by RNase	
	Control	FU
Endogenous RNase	15·2 ± 3·6	11·2 ± 1·8
Exogenous RNase	45·0 ± 7·9	56·0 ± 2·0

Control and FU extracts were prepared as described in Fig. 1. Each extract was divided into three parts. One part was used as control and stored in the cold without incubation. A second part received no RNase and served as a measure of endogenous RNase activity. The third part was treated with 4·6 units of RNase A. The latter two samples were incubated at 37° for 30 min and immediately layered on top of sucrose gradients and centrifuged for 110 min as described in Methods. The data represent the average of three experiments ± SEM for each treatment. The per cent degradation of ribosomes was calculated by determining the total radioactivity in the ribosomal fraction of the control minus the treated divided by the control times 100.

of RNase, respectively, per the 40 μg protein in each fraction. Fraction 3 received 4·6 units of RNase A. Fractions 2 and 3 were incubated for 30 min in a water bath at 37° with constant agitation, and all three fractions were immediately layered on top of sucrose gradients and centrifuged, as described in Methods. Table 1 shows that the breakdown of ribosomes due to endogenous or exogenous RNase was similar in control and FU-treated extracts. This data was taken to indicate that the RNase sensitivity of ribosomes from extracts of FU-treated cultures had not been dramatically increased.

Stability of ribosomes synthesized in presence of FU. To determine whether this reduction in labeling of the ribosomal fraction resulted from inhibition of ribosomal synthesis or from the destruction of ribosomes formed in the presence of FU, cultures were pulse labeled with [^{3}H]guanosine and the radioactivity in the ribosomal fraction was determined. Table 2 shows that incorporation of isotope into the ribosomal fraction was depressed by FU. When a FU culture was given a pulse of [^{3}H]guanosine, incorporation into the ribosomal fraction was inhibited (45·8 per cent). When a similar culture was allowed to grow for one doubling time the per cent [^{3}H]guanosine

was inhibited slightly more (54·4 per cent). For comparison the data from cultures continually labeled with [^{14}C]guanine for one doubling of turbidity are included. An average of 49·8 per cent inhibition of ribosomal labeling was observed in this culture. Thus, with pulse-labeled cultures sampled immediately or after a period of growth, or with continuous labeling throughout one doubling of turbidity, the per cent of total incorporation into the ribosomes was similar. Therefore, reduction in labeling of the ribosome fraction resulted from inhibition of ribosome synthesis and not the destruction of ribosomes formed in the presence of FU.

TABLE 2. EFFECT OF TIME OF LABELING ON DISTRIBUTION OF RADIOACTIVITY IN RIBOSOMAL FRACTION*

	Per cent of label recovered in ribosomal fraction		
	Control	FU	Inhibition (%)
Continual labeling, harvested after one doubling of A_{540}†	64·3	32·3	49·8
3 min pulse, no additional growth‡	64·6	35·0	45·8
3 min pulse, harvested after one doubling of A_{540}§	66·0	30·0	54·5

* Extracts were prepared and sucrose density gradient analysis was performed after centrifugation for 110 min as described in Methods. The results presented are the average of two experiments.

† [8-^{14}C]Guanine (20 nc/3·3 μg/ml) was added to each flask and the cultures were grown for one doubling of turbidity. Isotope incorporation continued throughout the course of incubation.

‡ [8-^{3}H]Guanosine (100 nc/5·3 ng/ml) was added to each flask 5 min after the addition of FU or water and the cultures were harvested 3 min after the addition of guanosine.

§ [8-^{3}H]Guanosine (100 nc/5·3 ng/ml) was added as in ‡ but the cultures were allowed to grow for one doubling of turbidity (Control, 50 min; FU, 120 min).

TABLE 3. EFFECT OF TEMPERATURE AND FU CONCENTRATION ON DOUBLING TIME AND RIBOSOME SYNTHESIS IN *B. cereus**

Incubation temperature	37°					27°	
FU (μM)	0	16	48	80	160	0	48
Doubling time (min)	50	105	120	140	180	105	220
% of ^{14}C in ribosomes	63·2	45·8	30·1	24·8	17·2	65·8	34·8

* [8-^{14}C]Guanine (20 mμc/3·3 μg/ml) was added to each flask and cultures were grown for one doubling of the turbidity at 540 nm. Extracts were prepared as described in Methods. Density gradient analysis was performed after centrifugation for 110 min as described in Methods. Results are the average of two determinations.

Effect of FU and temperature on ribosome biosynthesis and subcellular distribution. Since Ecker and Schaechter[14] observed that the proportion of ribosomal RNA of *Salmonella typhimurium* decreased as the doubling time increased, a correlation between FU concentration, doubling time and inhibition of ribosomal synthesis was desired. It can be seen in Table 3 that an inverse relationship existed (correlation coefficient = 0·969) between the doubling time of the FU-treated culture and the degree of ribosomal labeling with [^{14}C]guanine. Therefore it was essential to determine if the reduction in ribosomal labeling observed following FU treatment was merely a reflection of delayed growth or if it was a primary effect of FU.

In order to distinguish between increased doubling time and the effect of FU, experiments were conducted at 27°. Table 3 shows that the doubling time of control cultures was 50 min when grown at 37° and was increased to 105 min when grown at 27°. Nevertheless, ribosomal labeling was qualitatively and quantitatively identical. FU-treated culture (48 μM) whether grown at 27 or 37° exhibited similar ribosomal labeling even though doubling time was appreciably different (120 and 220 min, respectively). Thus, the doubling time itself had very little influence on the proportion of label incorporated into ribosomes in *B. cereus*.

In vitro *dissociation of ribosomes.* In order to determine the stability of ribosomes grown in the presence of FU, extracts of control and FU-treated cells (48 μM) were prepared in buffer containing 10 mM Mg^{2+} and then dialyzed for 6 hr against 500 vol. of buffer containing 0·1 mM Mg^{2+}. The dialyzed extracts were layered over a 5–20 per cent sucrose gradient and centrifuged for 3 hr as described in Methods. Under these conditions, extracts from control cells (Fig. 3a) contained the normal 50S and 30S ribosomal subunits and the 4S component as evidenced by both absorbancy and isotopic patterns. In contrast, the absorbancy pattern of extracts from FU-treated culture (Fig. 3b) showed only a small 50S and 30S peak and a very large 4S peak. The isotopic distribution which corresponds to the distribution of RNA synthesized in the presence of FU shows only a trace of the 50S and 30S materials. This is in contrast to the relatively high concentrations of 50S and 30S material seen in Fig. 1b at 10 mM Mg^{2+} and suggests instability of the products synthesized in the presence of FU.

To determine the fate of the 50S and 30S material synthesized in the presence of FU, a culture prelabeled with [^{3}H]guanosine was treated with 48 μM FU and [8-^{14}C] guanine. An extract of this culture was prepared in 10 mM Mg^{2+} buffer and sucrose density fractionation was performed. Tubes 11–13 were pooled as were tubes 18–20 and used as enriched 50S and 30S fractions, respectively. These fractions were dialyzed against 10 mM or 0·1 mM Mg^{2+} buffer and sucrose density gradient fractionation was again performed. When tubes 11–13 were analyzed once more at 10 mM Mg^{2+}, they were found to contain 70S, 50S and 30S ribosomes prelabeled during control growth [^{3}H] and predominantly a 50S particle labeled in the presence of [^{14}C]FU (Fig. 4a). When the Mg^{2+} concentration was 0·1 mM during dialysis (Fig. 4c), the 70S ribosome labeled during the control period of growth [^{3}H] dissociated to give 50S and 30S subunits and these subunits were stable. In contrast, the 50S particle synthesized in the presence of [^{14}C]FU was broken down to smaller particles. Figure 4b shows that tubes 18–20 contained 30S particles and a smaller 4S peak corresponding to material synthesized during control and FU-treated periods of growth. When the Mg^{2+} concentration was lowered to 0·1 mM, the 30S ribosomal subunit [^{3}H] was stable (Fig. 4d). In contrast, the 30S material synthesized in the presence of [^{14}C]FU was somewhat degraded to smaller particles. Thus, it was seen that ribosomal subunits synthesized during control periods of growth were stable when the Mg^{2+} concentration was lowered from 10 mM to 0·1 mM. In contrast, the 50S and 30S material synthesized in the presence of FU was found to be unstable under these conditions.

(2) *The effect of FU on RNA synthesis*

Preferential incorporation of FU into sRNA. In order to detect whether FU was preferentially incorporated into a subcellular fraction of *B. cereus*, [^{32}P]phosphate was added to a culture to label the new RNA, and [^{14}C]FU (final concentration 48 μM)

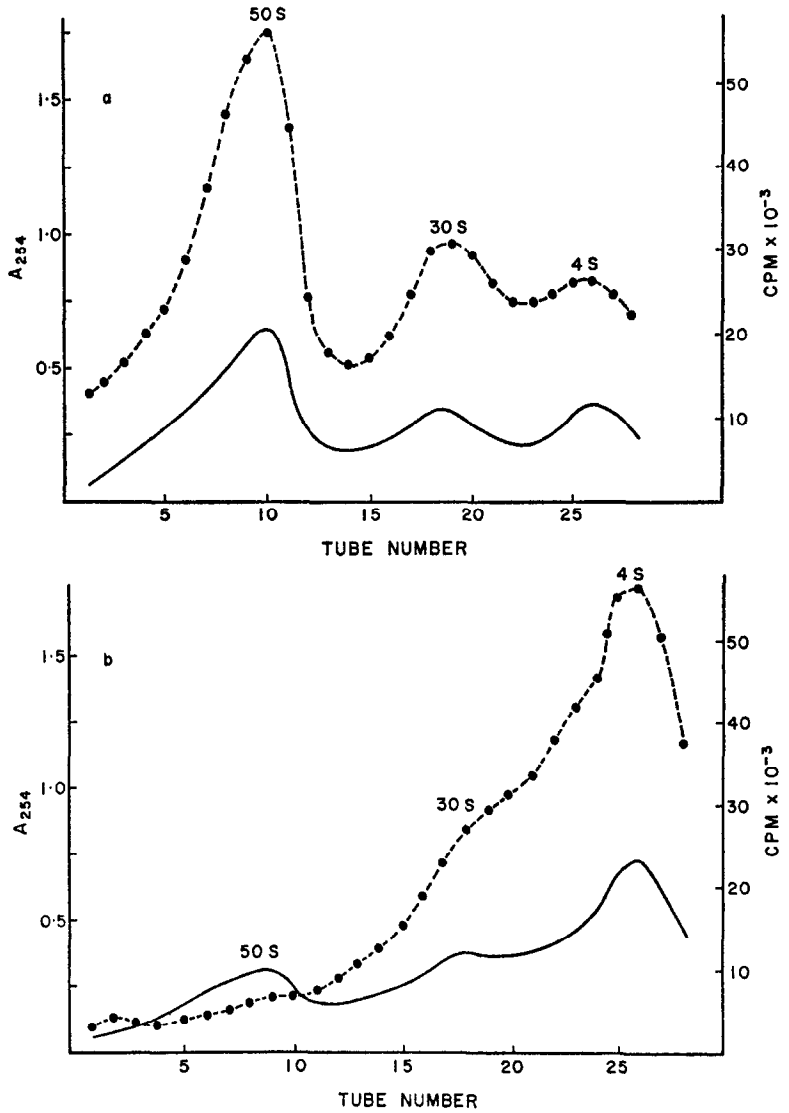

FIG. 3. Sucrose-density gradient analysis of extracts from control and FU-treated cells, but extracts treated at 0·1 mM Mg^{2+}. Conditions are as described in Fig. 1 except that the extracts were dialyzed in 500 vol. of buffer containing 0·1 mM Mg^{2+} and centrifuged for 3 hr. (a) Control, (b) 48 μM FU. Absorbancy at 254 nm, ———; CPM, – – – –.

was added to determine the distribution of FU in the newly synthesized RNA. The doubly labeled culture was harvested after one doubling of turbidity. Density gradient analysis of this culture is presented in Fig. 5. The density gradient profile of this culture demonstrated the lowered incorporation of RNA precursors [^{32}P] into ribosomal fractions that was observed in Fig. 1b. The distribution of [^{14}C]FU favored the 4S fractions even more, 66·7 per cent of the ^{32}P was found in the soluble fraction, whereas 71·4 per cent of the total [^{14}C]FU was recovered in this fraction. This difference in percentage of [^{14}C]FU and ^{32}P found in the soluble fraction (3·9 $\pm$ 0·8) was found to be statistically significant by the method of paired comparison ($P < 0{\cdot}05$;

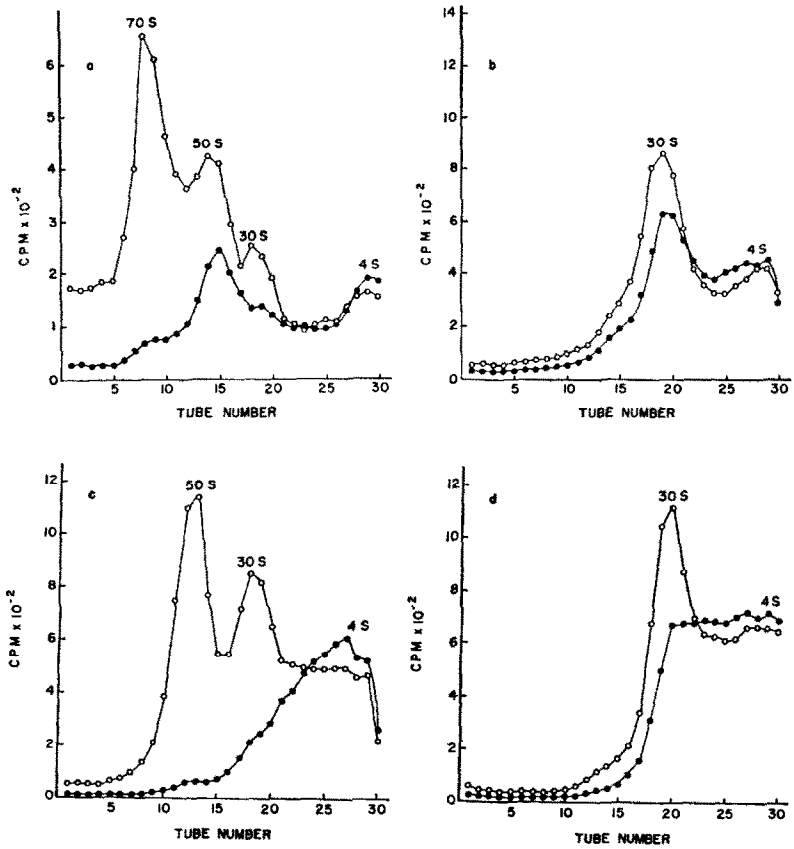

FIG. 4. Effect of 0·1 mM Mg^{2+} on ribosomal material synthesized in the presence of FU. A culture was prelabeled with [8-^{3}H]guanosine and subsequently labeled with [8-^{14}C]guanine after the addition of 48 μM FU. The culture was harvested after one doubling of turbidity and extract was prepared. The extract was fractionated after sucrose density gradient centrifugation in 10 mM Mg^{2+} as described in Fig. 1. Tubes 11–13 were pooled as were tubes 18–20 and were used as enriched 50S and 30S fractions, respectively, and were dialyzed against 10 mM or 0·1 mM Mg^{2+} buffer for 6 hr. Sucrose-density gradient centrifugation was done as described in Fig. 3. [^{3}H]CPM, ○; [^{14}C]CPM, ●. (a) Tubes 11–13 in 10 mM Mg^{2+}; (b) Tubes 18–20 in 10 mM Mg^{2+}; (c) Tubes 11–13 in 0·1 mM Mg^{2+}; (d) Tubes 18–20 in 0·1 mM Mg^{2+}.

N = 3). When the FU concentration was raised to 160 μM, the corresponding percentages of radioactivity recovered in the soluble fraction for ^{32}P and [^{14}C]FU labeling were increased to 81·7 and 90·7 respectively. This preferential incorporation of FU into the 4S fraction was also observed when RNA preparations rather than total cell extracts were examined.

Effect of FU on subcellular RNA synthesis. To observe the changes in RNA profiles due to the presence of FU, RNA was prepared from logarithmically growing cultures that had been labeled with [^{14}C]guanine for one doubling of turbidity in the presence or absence of 48 μM FU. The profile of control RNA (Fig. 6a) was typical of bacterial RNA and contained 23S and 16S rRNA and 4S RNA. The distribution profile of FU-RNA (Fig. 6b) differed from that of the control in that the relative quantity

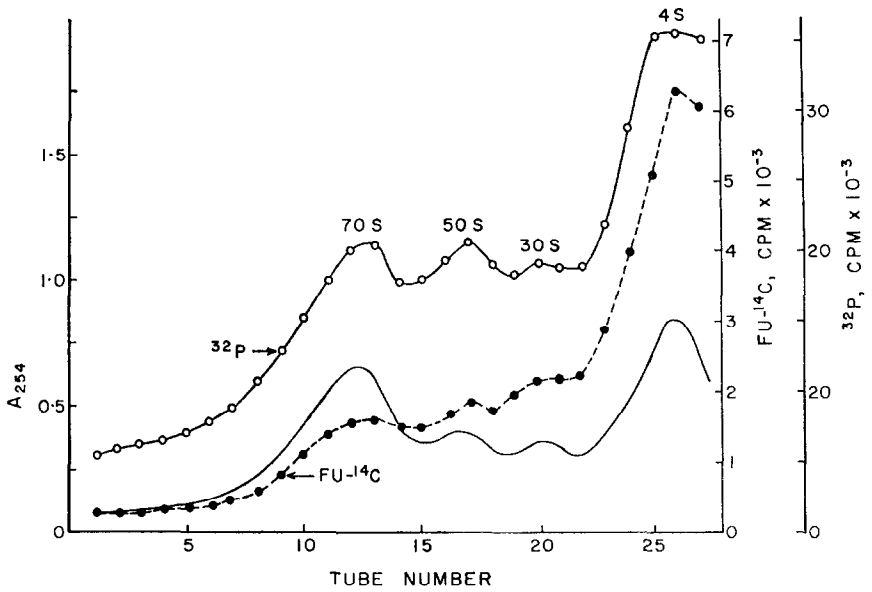

FIG. 5. Preferential incorporation of FU into soluble RNA. The culture was incubated in the presence of 48 μM [^{14}C]FU (2·5 μc/mg) and 0·1 μc ^{32}P/ml. After one doubling of turbidity, the culture was harvested and the extract prepared as described in Fig. 1. It was layered over 5–20 per cent sucrose gradient and centrifuged for 110 min as described in Methods. Absorbancy at 254 nm, ———; CPM, [^{14}C]FU, ●; CPM ^{32}P, ○.

of rRNA was reduced considerably. Furthermore, the height of the 23S RNA peak was less than that of the 16S RNA. A larger quantity of label was observed as 4S RNA in FU-treated cells, as had been noted earlier. It appears that incorporation of RNA precursors into the 23S rRNA was inhibited to a greater extent than incorporation into the 16S rRNA.

Role of RNase on stability of rRNA during FU-treatment. Additional experiments were performed to determine if this apparent decrease in labeling of 23S RNA was due to degradation by RNase in FU-treated cells or was related to reduced biosynthesis of this RNA species. A culture was prelabeled with [^{3}H]guanosine as described in Methods. At A_{540} of 0·1 the culture was divided into two portions. Both, culture A (control) and culture B (48 μM FU), were labeled with ^{32}P (0·1 μc/ml culture) for one doubling of turbidity. RNA was prepared from these cultures and the relative distribution of radioactivity in subcellular RNA fractions was calculated after density gradient fractionation. A summary of the fractionation results of the cultures is seen in Table 4.

In culture A (control) the ratio of ^{3}H to ^{32}P was constant in all RNA fractions, demonstrating that during these two periods of normal logarithmic growth the distribution of the two isotopes was identical. In contrast, in culture B (FU-treated) the ratio of ^{3}H to ^{32}P was increased, demonstrating, as did Fig. 5b, that rRNA synthesis was inhibited in cultures treated with FU. When the ^{3}H radioactivity of culture A (labeled during control growth followed by another period of control growth) was compared to the ^{3}H radioactivity of culture B (labeled during control growth followed by growth in presence of 48 μM FU), a statistically significant depres-

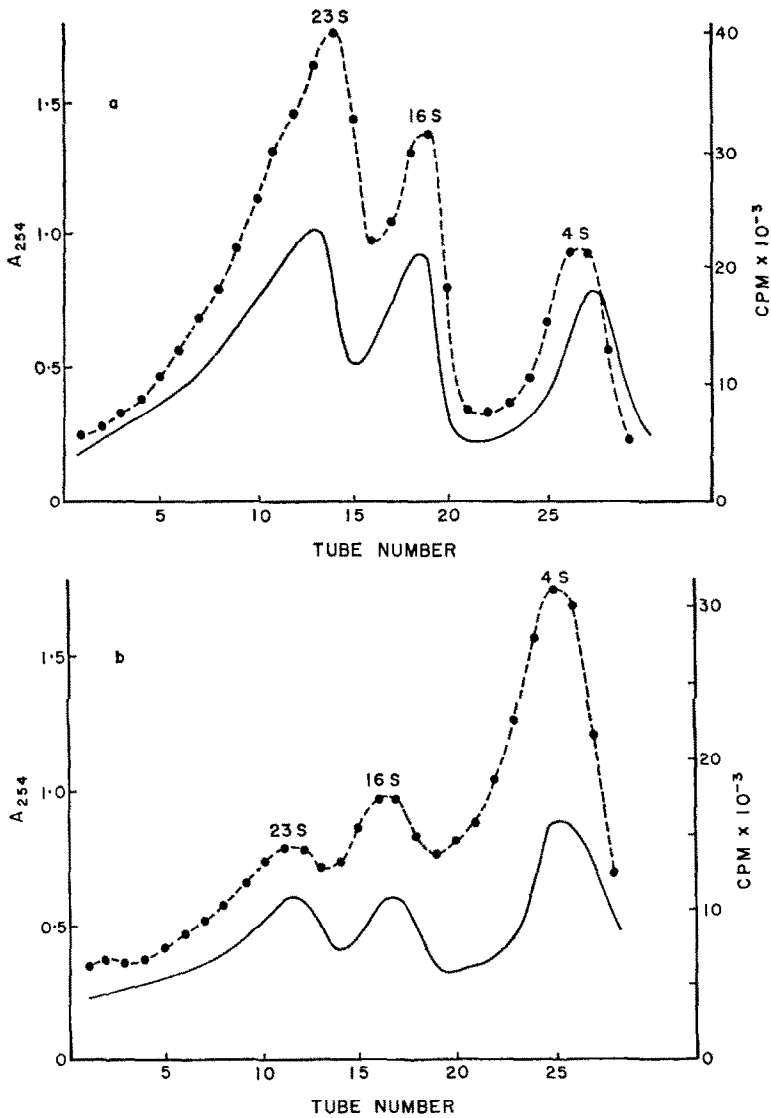

FIG. 6. Sucrose-density gradient analysis of RNA from control and FU-treated cells. Conditions as described in Fig. 1, and RNA was prepared as described in Methods. The RNA solutions were layered over 5–20 per cent sucrose and centrifuged for 4 hr as described in Methods. (a) Control, (b) 48 μM FU. Absorbancy at 254 nm, ———; CPM, – – – –.

sion in label in the 23S RNA fraction was observed ($P < 0{\cdot}001$; $N =$ six pairs) These data show that control 23S RNA is broken down either during growth in presence of FU or during isolation, whereas the 16S RNA fraction was not.

3. *The effect of FU on the interaction between protein and FU-containing RNA*

Effect of FU on relative RNA-protein composition of subcellular components. Shimura, Moses and Nathan[15] showed that bacteriophage MS2 synthesized in the presence of FU are deficient in RNA. We determined the A_{260}/A_{280} ratio (a crude measure

TABLE 4. EFFECT OF FU TREATMENT ON DISTRIBUTION OF RADIOACTIVITY IN rRNA

Culture	Isotope	rRNA	
		23S	16S
A	^{3}H	41·7 ± 4·9	26·6 ± 5·7
	^{32}P	40·8 ± 3·7	27·5 ± 4·3
	$^{3}H/^{32}P$	1·02	0·97
B	^{3}H	29·0 ± 5·3	26·0 ± 4·7
	^{32}P	10·2 ± 2·6	11·8 ± 3·1
	$^{3}H/^{32}P$	2·84	2·20
A − B	^{3}H	12·7 ± 1·5	0·6 ± 4·7
P		< 0·001	NS

Data represent the average of six paired experiments ± S.E.M. Statistical analysis was performed by method of paired comparison. "Untreated" culture A: first labeled with [^{3}H]guanine, then resuspended and labeled with ^{32}P. Culture B first labeled with [^{3}H]guanine as above, then resuspended and labeled with ^{32}P in the presence of FU.

of the ratio of RNA to protein) of density gradient fractions from control and FU-treated cultures. No gross alterations in this ratio were observed as a result of FU treatment. However, to determine more precisely whether an altered RNA to protein ratio occurred in subcellular fractions of FU-treated cultures, we prepared control and 48 μM FU-treated cultures and treated them with [^{14}C]leucine (to label protein) and ^{32}P (to label RNA). For the purpose of analysis, the gradients were divided into four fractions, and the statistics were performed by the method of paired comparisons. Thirty samples were collected from each gradient. The last eight tubes were defined as the soluble fraction (4S). Due to slight variation in the position of the 70S ribosomal peak among the experiments, the 70S ribosomal peak in each pair of experiments included the fraction of the control gradient with the greatest ^{32}P CPM and the three fractions, both preceding and following this tube. The ribosomal subunit fraction consisted of the fractions between the 70S ribosomal fraction and the soluble fraction. The polysomal fraction was made up of fractions that sedimented faster than the 70S ribosomal fraction. The results of five pairs of experiments are seen in Table 5.

FU produced pronounced reduction in the labeling of RNA in the polysomal and 70S ribosomal region with a concomitant increase in the soluble fraction. FU also produced a reduction in labeling of protein in the 70S ribosomal region with a relative increase in the soluble region. In addition, FU decreased the ratio of labeled RNA to labeled protein in the 70S ribosomal region and increased this ratio in the soluble region.

Thermal denaturation of ribosomes. To determine whether the differential incorporation of precursors into RNA and protein was associated with alterations in other properties of whole ribosomes, the thermal denaturation profiles of ribosomes were investigated.

Ribosomes were isolated, as described in Methods, from cells grown for one doubling of turbidity in the presence and absence of FU, and the melting temperatures (T_m) and hyperchromicity were determined. As can be seen in Fig. 7, the absorbance of

TABLE 5. EFFECT OF FU ON DISTRIBUTION OF ^{32}P AND [^{14}C]LEUCINE*

Culture	Polysomes	70S Ribosomes	Ribosomal subunits	Soluble
		% of ^{32}P		
Control	25·0 ± 3·8	36·1 ± 2·9	14·2 ± 1·6	24·3 ± 2·4
FU	9·1 ± 1·4	18·5 ± 1·9	22·1 ± 4·5	50·1 ± 2·8
P	< 0·025	< 0·025	NS	< 0·001
		% of ^{14}C		
Control	24·0 ± 3·1	23·1 ± 1·2	13·6 ± 2·3	39·1 ± 2·0
FU	17·8 ± 5·4	16·3 ± 1·0	17·9 ± 4·5	47·8 ± 3·1
P	NS	< 0·025	NS	< 0·01
		$^{32}P/^{14}C$		
Control	1·07 ± 0·17	1·56 ± 0·04	1·11 ± 0·13	0·62 ± 0·05
FU	0·76 ± 0·20	1·13 ± 0·05	1·29 ± 0·09	1·06 ± 0·07
P	NS	< 0·005	NS	< 0·001

* Parallel cultures were incubated with and without 48 μM FU. Each flask contained 0·1 μc ^{32}P/ml and 0·1 μc [^{14}C]leucine/ml. After one doubling of turbidity, cells were harvested and extracts prepared in 10 mM Mg^{2+} buffer. They were layered over 5–20 per cent sucrose gradient and centrifuged for 110 min as described in Methods. Samples were pooled as described in Results.

Amount of material found in each fraction is expressed as per cent of total label recovered ± SEM after density gradient centrifugation.

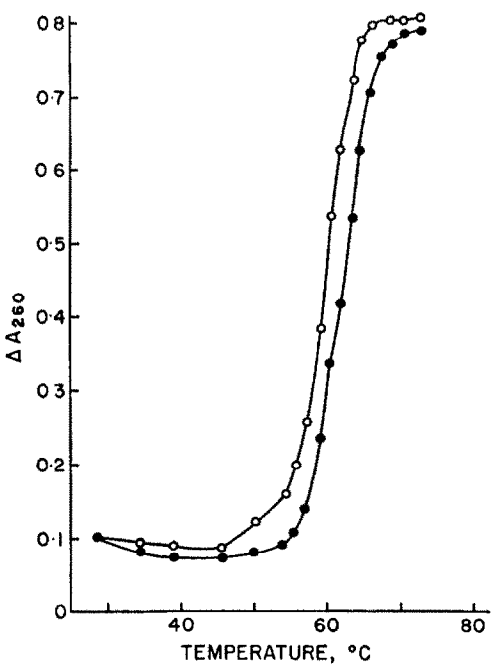

FIG. 7. Thermal denaturation of ribosomes. Ribosomes were prepared from control and 48 μM FU-treated cultures after one doubling of turbidity. Ribosomes were suspended in 10 mM Mg^{2+} buffer and treated as described in Methods. Initial absorbancy, Control (A_{260} = 0·750), ●; FU (A_{260} = 0·795), ○.

TABLE 6. THERMAL DENATURATION OF RIBOSOMES*

Experiment	T_m			Hyperchromicity (% increase A_{260})		
	Control ribosomes	FU ribosomes	ΔT	Control ribosomes	FU ribosomes	Δ%
1	68·5	65·5	3·0	62·8	47·6	15·2
2	70·2	68·0	2·2	56·4	41·4	15·0
3	73·2	71·8	1·4	59·6	66·0	−6·4
4	72·8	71·0	1·8	61·2	65·6	−4·4
5	62·3	60·5	1·8	53·9	60·7	−6·8
6	61·7	58·2	3·5	46·7	43·4	3·3
Mean	68·1 ± 2·1	65·8 ± 2·3	2·3 ± 0·3	56·8 ± 2·4	54·1 ± 4·6	2·6 ± 4·2
P			< 0·001			NS

* Ribosomes were prepared and treated as described in Methods.

FU-treated ribosomes increased at a slightly lower temperature than control ribosomes, but both sets of ribosomes increased in absorbancy to about the same extent. However, the observed difference in T_m was found to be significant (Table 6), whereas no statistically significant difference could be demonstrated for hyperchromicity between ribosomes from control and FU-treated cultures.

DISCUSSION

(1) *Effect of FU on ribosomes*

Previous investigations from this laboratory have shown that total RNA synthesis was unaffected by FU when cultures of the same turbidity were compared.[7] From the experiments presented here (Figs. 1, 6) it is clear that FU altered the subcellular composition of RNA since ribosomal RNA decreased, whereas 4S RNA increased concomitantly. These observations could be explained by either synthesis and subsequent breakdown of the ribosomes or by lack of synthesis of ribosomes.

No evidence of breakdown of RNA was observed in prelabeled control cultures as evidenced by the constancy of radioactivity during growth in the presence of FU. Additionally, it was demonstrated (Fig. 2b) that no breakdown of the RNA and subsequent reutilization of isotope had occurred during growth in the presence of FU. If significant breakdown and reutilization had occurred during growth in the presence of FU, the radioactivity profile would have shown relative increases in 50S, 30S and 4S peaks compared to the 70S ribosome peak. Therefore, it is concluded that ribosomes from control cultures are stable to growth in the presence of FU.

The decreased labeling of ribosomes observed after FU-treatment may be explained either by increased sensitivity to RNase or reduced synthesis of ribosomes. The data in Table 1 do not unequivocally demonstrate that ribosomes from FU-treated cultures do not have an increased susceptibility to RNase. The results indicate that a shift from 0° to 37° does not result in an appreciably higher degradation of ribosomes from FU-treated cultures than from control cultures. It is possible that degradation of the ribosomes from FU-treated cultures may have proceeded to a certain extent at 0°. However, the sensitivity of the ribosomes from FU-treated cultures would have to be increased by 50 per cent to account for the observed reduction in ribosomal

labeling, and no such increase is apparent. It was further demonstrated that ribosomes synthesized during the early period of FU inhibition were stable to subsequent growth in the presence of FU (Table 2). If the reduction in ribosomal labeling observed had been due to RNase activity or increased sensitivity of the ribosomes to RNase, it would have been manifest by a reduction in labeling observed in this preparation. Thus, because of the similar stability of control and FU-ribosomes and the lack of effect of continued growth in the presence of FU on ribosomes synthesized in the presence of FU, it was concluded that the diminished ribosomal labeling in the presence of FU was due to an inhibition of ribosomal synthesis.

The relative difference in distribution of ^{32}P and [^{14}C]FU (Fig. 5) suggests a preferential localization of FU in the 4S fraction. This preferential incorporation of [^{14}C]FU into the 4S fraction is unexplained.

Previous investigations from this laboratory have demonstrated that the growth inhibition produced by FU in cultures of *B. cereus* could be dissociated from inhibition of DNA synthesis.[7] The results presented in Table 3 demonstrate a direct relationship between growth inhibition produced by FU and the inability of cells to produce normal ribosomes. These results are interpreted to mean that the decreased ribosomal labeling observed was specifically related to FU, since another means of slowing growth, i.e. lowering the incubation temperature, did not decrease ribosome labeling.

(2) *Effect of FU on rRNA*

Although it appeared that large quantities of 50S and 30S subunits were synthesized in the presence of FU (Fig. 1), lowering of the Mg^{2+} concentration demonstrated that the 50S material and, to a lesser extent, the 30S material were not as stable as control 50S and 30S ribosomal subunits (Fig. 4). This suggests that synthesis of the 23S RNA and, to a lesser extent, the 16S RNA was interfered with by FU. Analysis of RNA prepared from FU-inhibited cultures demonstrated that synthesis of the 23S RNA was inhibited to a greater extent than is the 16S RNA (Fig. 6). This observation is in contrast to the apparently normal ratio of 23S to 16S RNA usually observed in *E. coli* after treatment with FU.[4,5,16,17] However, two other groups have reported less 23S RNA than 16S RNA following FU treatment in *E. coli*.[18,19] The release of RNase due to FU-treatment was considered as an explanation of the apparent lack of synthesis of 23S RNA. It was found that in *B. cereus* pre-existing 23S RNA was degraded as a result of FU treatment (Table 4), probably as a consequence of increased RNase activity. In addition, it was shown that FU also produced an inhibitory effect on 23S and 16S RNA synthesis greater than that accounted for by endogenous RNase ($^{3}H/^{32}P$ ratio of culture B, Table 4). Therefore, it appears that synthesis of 23S and 16S RNA was inhibited by FU and the additional inhibition of 23S labeling observed in *B. cereus* RNA from FU-treated cultures was due to the action of endogenous RNase. Liberation of RNase may explain the variable effects reported by various investigators for 23S to 16S RNA in *E. coli*[4,5,16–19] following FU treatment.

(3) *Effect of FU on the interaction between protein and FU containing RNA*

Three observations suggest that the interaction of protein and RNA was altered in FU-treated ribosomes. (1) The reduced synthesis of 23S rRNA suggests that the 50S particle formed during FU inhibition (demonstrated following fractionation at 10 mM

Mg^{2+}) was not the normal 50S ribosomal subunit. Supporting this, we have demonstrated that this 50S material was unstable at 0·1 mM Mg^{2+}, in contrast to normal 50S ribosomal subunits (Fig. 4). A similar argument can be made for the 30S particle synthesized in the presence of FU. (2) The slight lowering of T_m of ribosomes from FU-treated cells (Table 6) suggested a less ordered interaction of RNA and protein in FU ribosomes. Interpretation of these results is somewhat difficult because of the complex interaction between protein and RNA and the presence of control ribosomes in any ribosomal preparation from FU-treated cultures. Control ribosomes would be expected to account for 67 per cent of the ribosomes isolated after one doubling of turbidity in the presence of 48 μM FU. The statistically significant difference in T_m of control and FU ribosomes suggests a major change in heat stability of the FU-ribosome that is masked by the presence of control ribosomes. (3) It has been previously demonstrated that total protein synthesis was not altered by FU.[7] We have presented evidence that the subcellular distribution of protein was altered (Table 5). The ratio of RNA to protein was significantly decreased in the 70S ribosomal fraction and was increased in the soluble fraction. Changes in the ratio were also suggested in the polysome and ribosomal subunit fractions although they were not statistically significant. These results may be interpreted as a deficiency in rRNA or as an excess of protein in the 70S ribosomal fraction. Experiments are currently in progress to determine if there is any difference in the qualitative and quantitative nature of the protein associated with rRNA as a result of FU treatment.

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