Effects of Ribonuclease T_1 on *Escherichia coli* Ribosomes†

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ABSTRACT: Purified *Escherichia coli* ribosomes were digested with a low concentration of a water-insoluble derivative of RNase T_1. Such a treatment resulted in an impairment of activity of these ribosomes to support poly(U)-directed [^{14}C]polyphenylalanine synthesis. The AA-tRNA and poly(U) binding activities were also reduced. The decrease in these activities was proportional to the duration of the enzymatic treatment. The kinetics of inactivation of these ribosomal particles were biphasic. The initial rate of inactivation was rapid followed by a slower rate, about 22% of the initial rate. The rate of inactivation of a mixture of 30S and 50S subunits was faster than that of the 70S particles. The RNAs remaining in the ribosomal particles after such an enzymatic treatment was analyzed by column chromatography on DEAE-cellulose at pH 6.5 and 2.7. Relatively few fragments were observed. The 3′-terminal oligonucleotides of the rRNAs which had been labeled with tritiated $NaBH_4$ were found among these protected RNA regions. Uridine was found to be the major terminus of these RNA fragments.

Ribosome plays an important role in the biosynthesis of proteins and can be considered as a multimacromolecular complex containing numerous active sites for the binding of various molecules (see review by Nomura, 1970). However, the role of rRNA in maintaining the ribosomal structure and function requires further elucidation. One experimental approach in studying the molecular organization of the ribosome is to digest the ribosomal particles with specific nucleases. In fact, several investigators reported that portions of the rRNA were inaccessible to pancreatic RNase whereas some of the RNAs in these ribosomal particles were degraded by the enzyme (Santer, 1963; Fenwick, 1968; Möller *et al.*, 1969; Cox, 1969; Godson and Cox, 1970; Ehresmann and Ebel, 1970; Huvos *et al.*, 1970). It was supposed that these accessible RNA regions were located at the surface of the particles. Such an experimental approach was used to obtain some information regarding the spatial arrangement between the RNA molecules and the large numbers of proteins of rabbit reticulocyte ribosomes (Cox, 1969). In addition, such a method was used to obtain larger RNA fragments for base sequence analysis (Santer and Szekely, 1971; Fellner *et al.*, 1970).

The aim of the present study is to examine the nature of the RNA regions of *Escherichia coli* ribosomal particles which are accessible to RNase T_1 digestion and to attempt to correlate the structure of these RNA regions to the various functions of the ribosomal particles in protein biosynthesis. A brief report of this work has been published (Lee and Quintanilla, 1971).

Experimental Section

Materials. Ribonuclease T_1 was purchased from Calbiochem, Los Angeles, Calif. [^{14}C]Amino acids and tritiated $NaBH_4$ were obtained from New England Nuclear Corp., Boston, Mass. Sepharose 2B and DE-52 were obtained from Pharmacia and Whatman, respectively. Radioactive [^{14}C]-poly(U) (molecular weight about 10^5) was purchased from Schwarz BioResearch, Inc., New York, and nonradioactive poly(U) (molecular weight about 10^5) was the product of Miles Laboratories, Elkhart, Ind.

Preparation of Ribosomes. E. coli A19 were grown to early log phase in nutrient broth (Gesteland, 1964). Cell extracts were prepared by grinding frozen cells with alumina and crude ribosomal particles were obtained by high-speed centrifugation (105,000*g*, 3 hr). These ribosomal particles were further purified by three cycles of washing and pelleting in ammonium chloride (Salas *et al.*, 1965). Ribosomal preparation obtained in this manner was checked in the analytical ultracentrifuge Beckman Model E and was shown to contain approximately 90% of 70S particles. Ribosomal subunits were obtained by dissociation of the purified 70S particles by dialysis in Tris buffer containing low concentration of magnesium (0.05 M Tris-HCl, pH 7.4, 10^{-4} M $MgCl_2$, and 0.06 M KCl) at 4° overnight. Only freshly prepared ribosomal samples were used in experiments reported in this paper.

Preparation of RNase T_1 Derivative. RNase T_1 was covalently linked to Sepharose 2B by published procedure (Lee, 1971) and was routinely checked for bound and residual unbound enzymatic activity. Only those preparations without detectable unbound enzymatic activity were used for these studies.

Ribonuclease Digestion of Ribosomes. Purified ribosomes (6 mg) in Tris-acetate buffer (2 ml, 10^{-2} M, pH 7.6 at 25°) containing magnesium acetate (10^{-2} or 10^{-4} M) were incubated with a very low concentration of the water-insoluble derivative of RNase T_1 [1.2 enzymatic units of activity (Lee, 1971)]. The mixtures were incubated at 37° for various times with very gentle stirring. Reaction was terminated by removing the enzyme derivative by centrifugation at full speed in a table model International clincial centrifuge at 4° for 1 min. Supernatant was withdrawn carefully leaving behind all the enzymatic derivative and was used immediately for assays of biological activities or for isolation of RNA. About 95% of the ribosomes were recovered after removal of the RNase.

Determination of Biological Activities of Ribosomal Particles. ABILITY TO SUPPORT PROTEIN SYNTHESIS. Cell-free amino acid incorporation system was prepared and the ability of the ribosomal particles to support poly(U)-dependent polyphenylalanine synthesis was tested essentially as described

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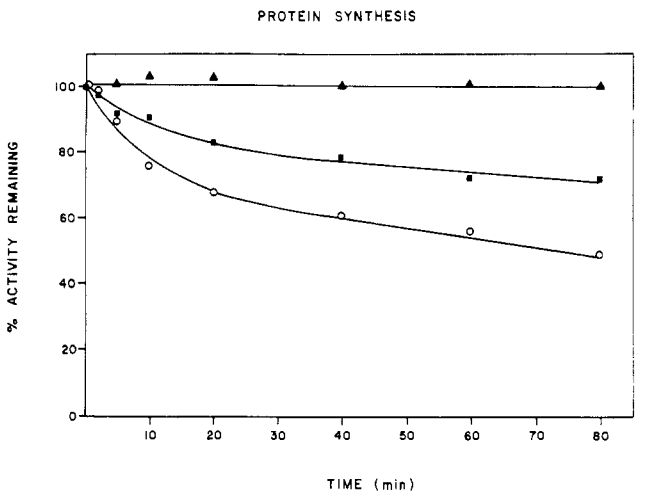

FIGURE 1: Ability of ribosomes to support poly(U)-directed [^{14}C]-polyphenylalanine synthesis. Ribosomes were treated with Sepharose–RNase T_1 for various times at 37°. After removing the RNase T_1 derivative, the ribosomes were incubated in the cell-free amino acid incorporation system for 20 min at 37°. Trichloroacetic acid was added; the precipitate was collected on Millipore filters and the radioactivity measured as described. (▲) Control 70S ribosomes, (■) RNase T_1 treated 70S ribosomes, and (○) RNase T_1 treated 50S and 30S subunits.

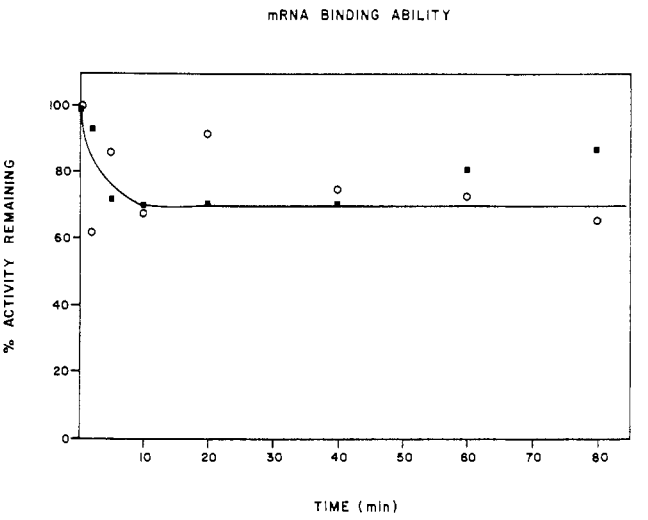

FIGURE 2: Ability of ribosomes to bind [^{14}C]poly(U). Ribosomes were treated with the RNase T_1 derivative as described in the Experimental Section. After removing the enzyme, ribosomes were tested for their ability to bind[^{14}C]poly(U) with the filtration method. (■) RNase treated 70S and (○) RNase-treated 50S and 30S subunits.

(Nirenberg, 1963). The reaction was terminated with cold trichloroacetic acid (final concentration 5%) after a 20-min incubation at 37°. The sample was kept in ice for 30 min and was heated for 20 min at 90°. The protein precipitate was filtered on Millipore filter, washed five times with cold Cl_3CCOOH (5%), dried under a heat lamp, and moistened with ten drops of NE219 scintillator. The radioactivity was determined using a Packard 3375 liquid scintillation spectrometer.

ABILITY TO BIND mRNA. Ribosomes were tested for their ability to bind [^{14}C]poly(U) with a filtration method essentially as described (Moore, 1966). Ribosomes were filtered on Millipore disks under reduced pressure at a moderate flow rate followed by a solution of bovine serum albumin (6 mg in 2.5 ml). A buffer solution (3 ml) containing [^{14}C]-poly(U) (1 μCi, 1 mg) was passed through followed by two washings of buffer. The filter disk was dried and the radioactivity was measured as described above.

ABILITY TO BIND AA-tRNA. [^{14}C]Phe-tRNA was prepared *in vitro* according to a published procedure (Ehrenstein, 1967). The AA-tRNA·ribosome binding assay of Nirenberg and Leder (1964) was used. Each reaction mixture in a total volume of 50 μl contained: Tris-acetate (0.05 M, pH 7.2), potassium acetate (0.05 M), magnesium acetate (0.03 M), 3.0 ODU at 260 mμ of ribosomes, [^{14}C]Phe-tRNA (0.5 ODU at 260 mμ), and poly(U) (0.1 ODU at 260 mμ). Reaction mixtures were incubated for 20 min at 25°. Ribosomes were then filtered on Millipore filters, dried, and the radioactivity was determined as described above.

Terminal Labeling of RNA with Tritiated $NaBH_4$. The procedure for labeling the 3′ termini of RNA chains was essentially that of RajBhandary (1968) and Leppla *et al.* (1968). The RNA was first dissolved in sodium phosphate buffer (0.01 M, pH 6, 0.2 ml); sodium periodate (10 μl, 0.05 M) was then added. The solution was left in the dark at room temperature for 1 hr. After the addition of NaOH (1 N, 10 μl), tritiated $NaBH_4$ (50 μl, 0.01 M) was added and left standing in a hood at room temperature in the dark for 1 hr. Excess borohydride was decomposed by the addition of acetic acid (30 μl, 1 N). The mixture was dried, dissolved in water, and dried again, *in vacuo*.

Isolation and Fractionation of RNA Fragments from Ribosomes. Polynucleotides from *E. coli* ribosomes were extracted by a previously described method (Lee *et al.*, 1970). After terminal labeling, the dried product was dissolved in 2.0 ml of Tris-chloride (0.01 M, pH 6.5), $MgCl_2$ (0.02 M), and NaCl (0.3 M) and absorbed to a DEAE-cellulose column (0.6 × 75 cm) which had been equilibrated with the same buffer. Elution was effected with 800 ml of buffer containing a linear gradient of NaCl (0.3–1.2 M) at a flow rate of 9 ml/hr. The elution was monitored by measurement of the optical density of the eluate at 260 mμ. At the end of the gradient, the column was washed with a solution of Tris-chloride (0.01 M, pH 6.5) containing $MgCl_2$ (0.02 M), NaCl (2 M), and urea (7 M).

The peak fractions were pooled and desalted by reabsorbing the nucleotide material onto a DEAE-cellulose column (bicarbonate form) and eluting with triethylamine bicarbonate as described (Lee and Ingram, 1969). Radioactivity present in each peak was measured by taking an aliquot of the sample, mixing it with 10 ml of Bray's solution (Bray, 1960), and counting in a Packard 3375 scintillation counter.

Further subfractionation of oligonucleotides was achieved by column chromatography on DEAE-cellulose at pH 2.7. The desalted oligonucleotide peak was dissolved in the starting buffer (2 ml) and applied to a DEAE-cellulose column (1 × 24 cm). Chromatography was effected with 400 ml of sodium citrate buffer (0.05 M, pH 2.7) containing a linear gradient of NaCl (0.1–0.6 M) at a flow rate of 6 ml/hr.

Base Ratio Analysis. The oligonucleotide was hydrolyzed to its constituent mononucleotides by alkaline hydrolysis (Lee and Gilham, 1966). The mixture was neutralized with perchloric acid and, after removing the precipitate, was analyzed by a modification of the method described by Cohn and Volkin (1951) using a column of Dowex 1-X2 formate, 200–400 mesh. After the elution of the nucleoside trialcohol derivatives with water, the nucleotides were eluted in the order Cp, Ap, Up, and Gp using the four solvents described (Cohn and Volkin, 1951).

Identification of Terminal Nucleosides. The water washing

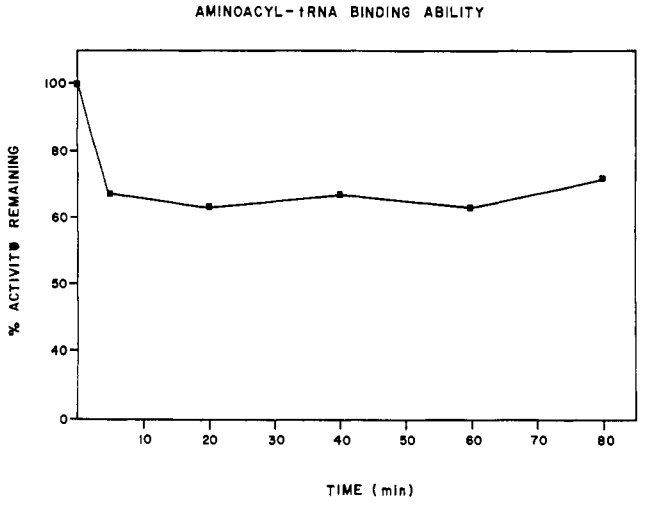

FIGURE 3: Ability of ribosomes to bind AA-tRNA. Ribosomes (70 S) which have been treated for various times with the RNase T_1 derivative were tested for their ability to bind [^{14}C]Phe-tRNA in the presence of poly(U) as described in the Experimenal Section.

from the column of Dowex 1-X2 formate was concentrated, mixed with 1 ODU each of the four nucleoside trialcohols, and applied quantitatively to Whatman No. 3MM paper. Descending chromatography was carried out in solvent B (DeWachter and Fiers, 1967) for 18 hr. Uv-absorbing spots were cut out and placed in counting vials with Bray's scintillator (10 ml). Radioactivity was measured with a Packard 3375 liquid scintillation counter.

Results

Effects of RNase T_1 on Biological Functions of 70S Ribosomes. Purified 70S ribosomal particles from *E. coli* A19 were digested at 37° with a low concentration of RNase T_1 in the presence of 10^{-2} M MgCl (pH 7.6). Aliquots were withdrawn at various times. After the enzyme derivative was removed by centrifugation, the treated ribosomes were tested for their ability to support protein synthesis in a cell-free amino acid incorporation system. As shown in Figure 1, nuclease digestion gradually reduced their ability to support poly(U)-directed [^{14}C]polyphenylalanine synthesis. The decrease in this activity was proportional to the duration of the enzyme treatment. The rate of inactivation of ribosomes by RNase T_1 was rather rapid for the initial 10 min of reaction and changed to a slower rate, about 22% of the initial rate. After 80 min of nuclease digestion, about 30% of the original biological activity was abolished. Control experiments in which ribosomal particles were incubated under similar conditions excluding RNase T_1 in the incubation mixture showed no loss of biological activity. In addition, the enzymatic activity of the ribonuclease derivative was measured at the end of the reaction. No loss in specific activity was detected. Thus, the observed decline in the inactivation of ribosomes could not be the result of loss in nuclease activity.

The ability of the treated ribosomes to bind synthetic mRNA was tested in an attempt to determine if this function of the ribosomes was being specifically affected by nuclease digestion. A typical plot showing the [^{14}C]poly(U) binding ability of such ribosomes is shown (Figure 2). It is clear that nuclease treatment resulted in a partial loss of poly(U) binding ability. The rate of inactivation of this particular function

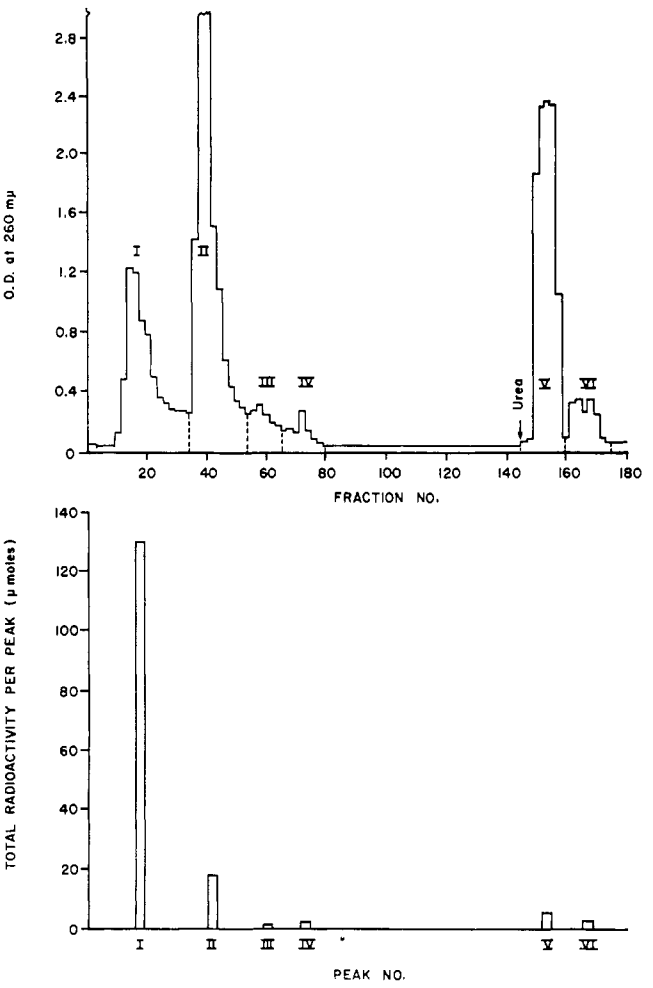

FIGURE 4: Elution pattern from the column chromatography of the RNA fragments from the 20-min ribosomal sample. The column consisted of DEAE-cellulose (Whatman DE-52; chloride form; 0.6 × 75 cm) and the elution was effected with 800 ml of 0.01 M Tris-Cl (pH 6.5), and 0.02 M $MgCl_2$ containing a linear gradient of NaCl (0.3–1.2 M) at a flow rate of 9 ml/hr. At the point indicated, the column was washed with buffer containing NaCl (2 M) and urea (7 M).

of the ribosomes correlated with the amino acid incorporation data. The 70S particles lost up to 30% of the mRNA binding ability in the first 10 min of incubation. However, no further significant loss in activity was observed after this initial time period.

The capability of these treated ribosomes to bind AA-tRNA in the presence of exogenous messenger was also examined. As shown in Figure 3 their ability to bind [^{14}C]Phe-tRNA in the presence of poly(U) was diminished. Again, up to 30% of the AA-tRNA binding ability was lost in the first 10 min of reaction and no further decrease was observed after this time.

Effects of RNase T_1 on the Biological Functions of 50S and 30S Ribosomes. A mixture of 50S and 30S ribosomal subunits was digested with RNase T_1 under conditions similar to those used for the digestion of 70S particles. At various times, aliquots were withdrawn and the biological activities of the samples were measured in the amino acid incorporation system. Figure 1 shows the activity of the ribosomal subunits as a function of nuclease treatment. They lost their biological activity more readily than the 70S particles, so that after 80 min of enzymatic treatment almost 50% of the

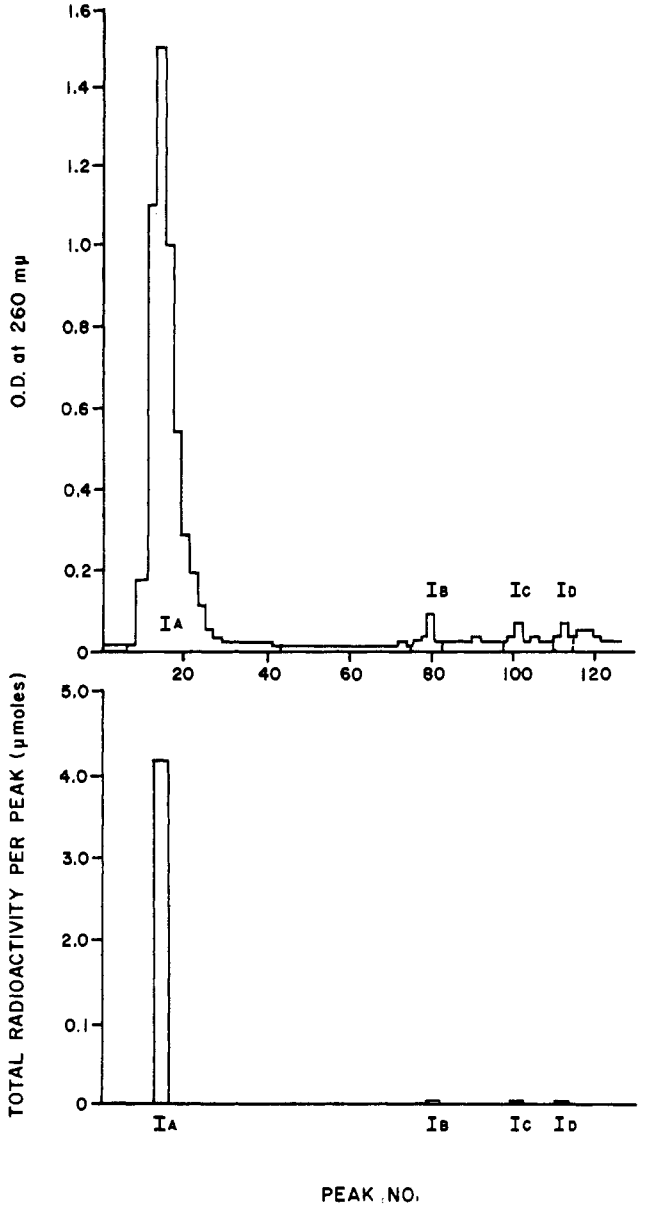

FIGURE 5: Elution pattern of peak I from the 20-min ribosomal sample on a column of DEAE-cellulose at pH 2.7. After removal of salts, material from peak I (Figure 4) was loaded onto a DEAE-cellulose column (DE-52; chloride form; 1 × 24 cm). Elution was effected with 400 ml of sodium citrate buffer (0.05 M, pH 2.7) containing a linear gradient of sodium chloride (0.1–0.6 M) at a flow rate of 6 ml/hr.

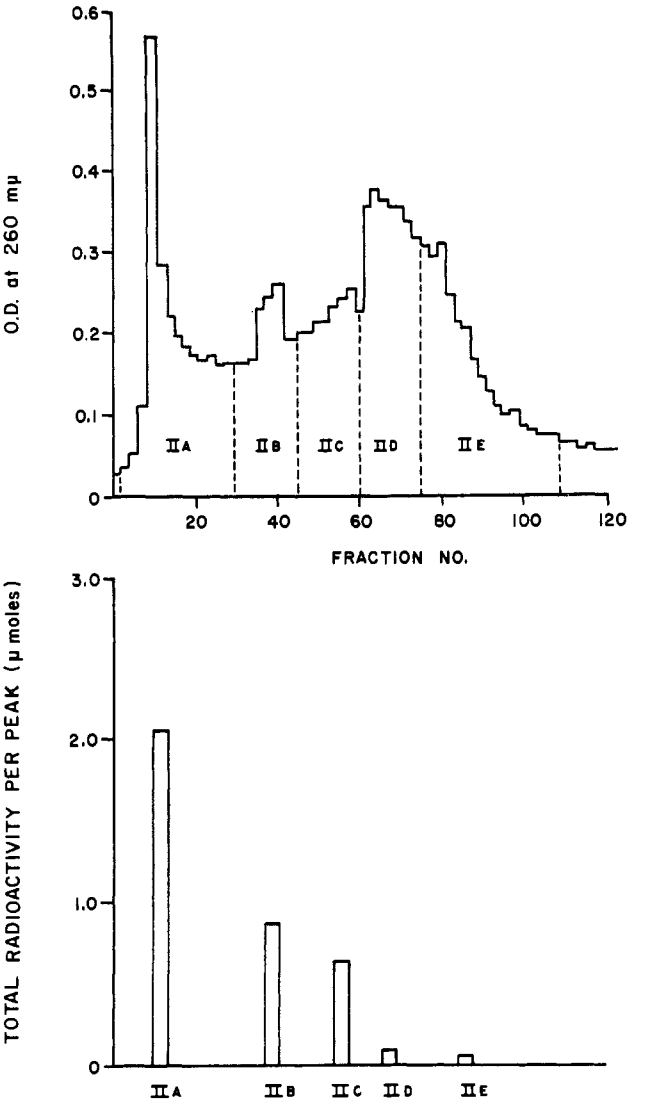

FIGURE 6: Elution pattern of peak II from the 20-min ribosomal sample on a column of DEAE-cellulose at pH 2.7. Conditions for the column chromatography were identical with those used for the fractionation shown in Figure 5.

original activity was lost. There appeared to be a change in the rate of inactivation at about 10 min of reaction; the initial rate was approximately sixfold faster than that after the 10-min point.

The capability of these RNase-treated ribosomal subunits to bind synthetic mRNA was also examined. The percentage of mRNA binding activity remaining after varying duration of nuclease digestion is shown in Figure 2. The data indicate that digestion by nuclease impaired their ability to bind [^{14}C]poly(U). Up to about 30% of the activity was abolished after the initial 10 min of incubation. No significant loss in activity was observed beyond the initial 10 min of incubation. The observed digestion kinetic for the 50S and 30S subunits was similar to that for the 70S particles.

Effects of RNase T_1 on the Structure of rRNA. LIMITED DIGESTION. To examine the conditions of the ribosomal RNA molecules in the 70S particles after RNase T_1 treatment, RNA molecules were isolated from 70S ribosomes which had been incubated with RNase T_1 for 20 min (see Figure 1; a 20-min sample with 85% activity remaining). The 3′-hydroxyl termini of the rRNA chains were labeled with radioactive sodium borohydride after periodate oxidation. The oligonucleotides were then chromatographed on a column of DEAE-cellulose at pH 6.5. A typical elution profile is shown (Figure 4). About 90% of the uv-absorbing material applied to the column was recovered. The radioactivity present in each peak is also shown. Peak I contained about 85% of the total radioactivity applied to the column and was further fractionated by column chromatography on DEAE-cellulose at pH 2.7. A typical elution profile is shown; only one fraction contained radioactivity (Figure 5). Of the material applied to the column, measured either by radioactivity or by uv, 92% was recovered.

Peak II which contained 11% of the total radioactivity applied to the column was similarly analyzed on a DEAE-cellulose column at pH 2.7. The elution profile and the radio-

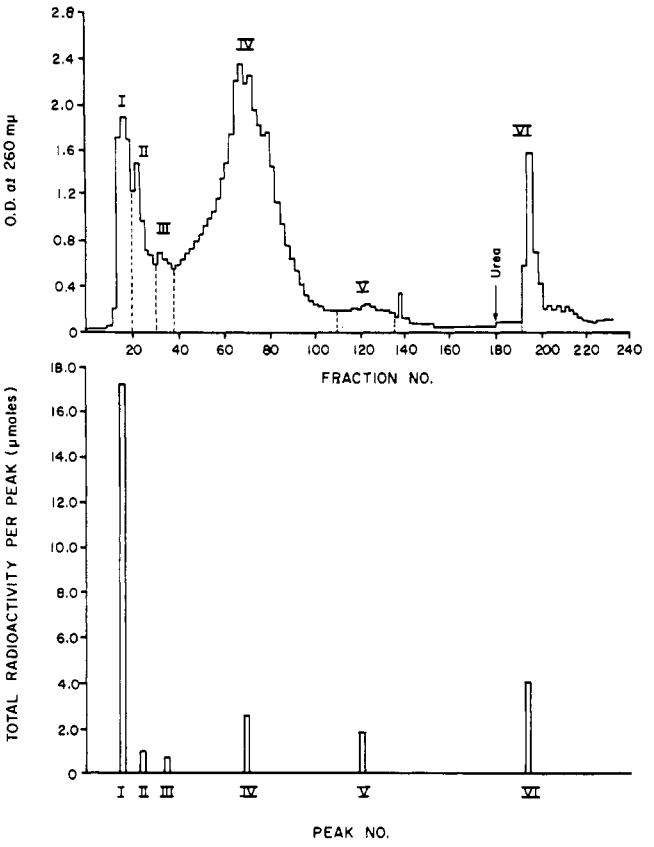

FIGURE 7: Elution pattern from the column chromatography of the RNA fragments from the 60-min ribosomal sample. The conditions for the column chromatography were identical with those used for the fractionation shown in Figure 4.

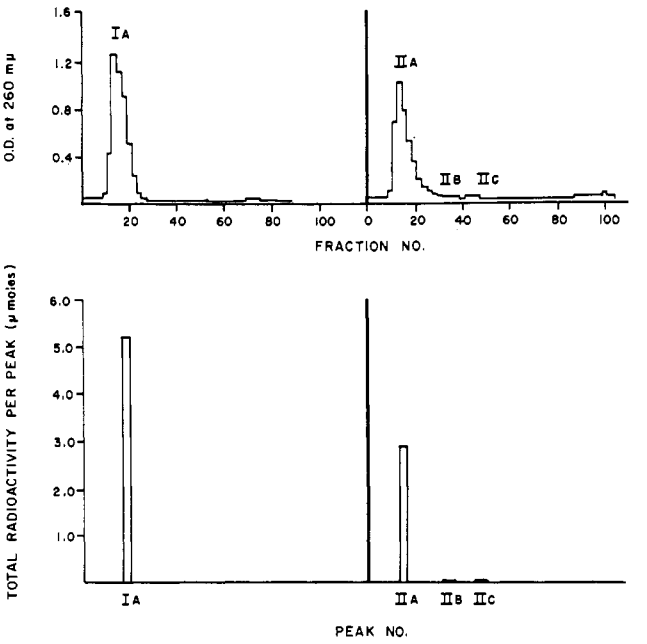

FIGURE 8: Fractionation of peaks I and II of the 60-min sample of RNA on DEAE-cellulose at pH 2.7. Conditions for chromatography were identical with those shown in Figure 5.

activity distribution pattern are shown (Figure 6). The elution profile was more complex than that of peak I and revealed at least five peaks. Peak II_A was the most highly labeled fraction (57%) but peak II_B and peak II_C also contained considerable amounts of radioactivity (23 and 17%, respectively).

MORE EXTENSIVE DIGESTION. RNA was isolated from ribosomes which had been treated with RNase T_1 for a longer period of time (60 min) and thus a greater extent of their biological activities was abolished (Figure 1; a 60-min sample with about 70% activity remaining). After labeling with sodium borohydride, the RNA sample was analyzed by column chromatography in a similar manner as described for the 20-min sample. The elution profile on a column of DEAE-cellulose at pH 6.5 is shown (Figure 7). Of the uv-absorbing material applied to the column, 87% was recovered. The radioactivity present in each peak is also shown. The profile is quite different from that of the 20-min sample. However, peak I was again the most highly labeled fraction containing about 50% of the total radioactivity applied to the column. Upon further purification of peak I on a column of DEAE-cellulose at pH 2.7, only one peak was obtained (Figure 8).

Peak II, containing approximately 3% of the total radioactivity, was also purified by column chromatography at acidic pH and only one peak contained radioactivity (Figure 8). Peaks III and IV containing 2% and 9% of the total radioactivity were similarly analyzed. The elution profiles are shown in Figure 9. Peak III was subfractionated into two peaks (III_A and III_B). Peak III_A was approximately 3.7 times more radioactive than peak III_B. Peak IV was resolved into three

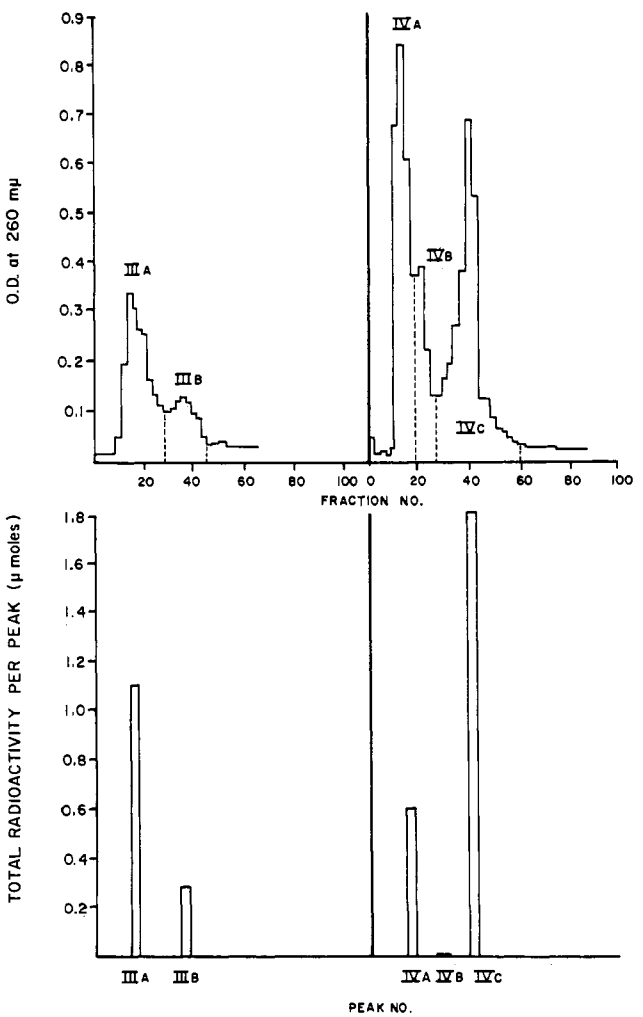

FIGURE 9: Fractionation of peaks III and IV of the 60-min sample of RNA on DEAE-cellulose at pH 2.7. Conditions for column chromatography were identical with those used for the fractionation shown in Figure 5.

TABLE I: Nucleotide Composition of RNA Fragments.

Peak	Mole % Ap	Gp	Cp	Up	G + C:A + U	Pu:Pyr	% G + C
20-min sample							
I_A	47.56	16.24	18.80	17.39	0.54	1.76	35.04
II_A	52.92	0	31.77	15.31	0.47	1.12	31.77
II_B	47.43	0	35.29	17.27	0.55	0.90	35.29
II_C	33.11	13.92	27.26	25.71	0.70	0.89	41.18
60-min sample							
I_A	30.30	21.39	23.52	24.79	0.82	1.07	44.91
II_A	49.49	10.98	21.33	18.20	0.48	1.53	32.31
III_A	56.42	0	27.74	15.84	0.38	1.29	27.74
IV_A	46.56	0	53.44	0	1.15	0.87	53.44
IV_C	40.82	0	31.20	27.98	0.45	0.69	31.20
23[a]	25.5	32.8	21.0	20.6	1.17	1.40	53.8
16[a]	24.5	31.6	22.5	21.3	1.18	1.29	54.1
5[a]	19.2	33.8	30.0	17.0	1.76	1.13	63.8

[a] The nucleotide composition of these RNAs are derived as an average from a number of literature data (Spirin and Gavrilova, 1969).

peaks, but the bulk of the radioactivity (94%) was found in two peaks.

BASE RATIOS OF LABELED RNA FRAGMENTS. To further assess the nature of these RNA segments, the most highly labeled fractions from the 20- and the 60-min samples, *i.e.*, those presumably generated from the original 3′-hydroxyl termini of the intact rRNA chains, were subjected to alkaline hydrolysis. The nucleotides present in each sample were analyzed on a column of Dowex 1-X2 formate. The radioactively labeled 3′-nucleoside derivative was eluted with water and was analyzed subsequently by paper chromatography (see below). The nucleotide composition of each of these RNA fragments is shown in Table I. The amount of radioactivity present in each nucleotide fraction was also measured. No significant amount of radioactivity was found in any nucleotides indicating that the sodium borohydride reduction occurred chiefly at the terminal 3′-nucleoside rather than at the nucleotides in the internal positions of the RNA chains. With the exception of fraction I_A from both the 20- and 60-min samples, fraction II_C from the 20-min sample and fraction II_A from the 60-min sample, these labeled RNA fragments do not contain detectable amounts of guanylic acid.

The G + C:A + U and the purine:pyrimidine ratios of each fragment are also shown (Table I) and in each case these ratios are quite different from those of the intact rRNA species. In general, these labeled fragments have lower percentages of guanosine–cytosine than the intact rRNA chains.

TERMINAL BASE CHARACTERIZATION. The tritium-labeled terminal nucleoside trialcohols present in each of these RNA fragments were analyzed by paper chromatography along with nonradioactive standards. In all cases, uridine trialcohol was the major component indicating that uridine was the terminus of these RNA fragments (Table II). However, in some cases, up to 30% of adenosine was detected.

TABLE II: Terminal Nucleoside of RNA Fragments.

Peak	A	G	C	U
20-min sample				
I_A	17	0	0	83
II_A	25	0	0	75
II_B	29	0	0	71
II_C	5	0	0	95
60-min sample				
I_A	22	0	0	78
II_A	15	0	0	85
III	0	0	0	100
IV_A	5	0	0	95
IV_C	30	0	0	70

Discussion

One of the major difficulties commonly encountered in the study of nuclease action upon ribosomes is that even after the termination of the nuclease treatment there is residual nuclease activity which can further attack the RNAs. We have circumvented this difficulty by covalently linking RNase T_1 to a water-insoluble support which can be separated from the substrate completely and rapidly at the end of the desired reaction. With the use of such an enzyme derivative we have studied the action of RNase T_1 on the functions and structure of *E. coli* ribosomes.

Our data indicated that *E. coli* ribosomes could be inactivated by the action of RNase T_1. The inactivation kinetics as shown in Figure 1 suggest either a heterogeneity of the ribosomal population in their sensitivity to nuclease digestion or an inactivation of the RNase derivative. Our finding that no intact 23S and 16S rRNA was detected in the nuclease digested ribosomes makes the former possibility unlikely. Although it is possible that a difference exists, but it is so small that it escapes our current means of analysis. The latter possibility can be eliminated based on our finding that the specific activity of the enzyme remained the same at the end of the incubation.

The rate of inactivation of a mixture of 30S and 50S subunits was faster than that of the 70S particles. Thus, our findings imply that certain segments of the rRNA molecules were involved in the association of the two subunits. These RNA regions were inaccessible to the nuclease when the ribosome was in the form of a 70S particle but became exposed when the ribosome existed in the dissociated stage. These RNA regions may be directly protected by some ribosomal proteins or perhaps by some steric hinderance due to the association of the subunits. However, it cannot be completely ruled out that a conformational rearrangement of the subunits might have occurred due to the association or dissociation process, although the studies of McPhile and Gratzer (1966) and Sarkar *et al.* (1967) made the latter possibility unlikely.

It is interesting to point out that our conclusion is in general agreement with those of Cox (1969) who reported that the ability of rabbit reticulocyte ribosomes to incorporate [^{14}C]-phenylalanine into acid-insoluble protein was diminished after treatment with low concentrations of pancreatic RNase. A similar conclusion was drawn from experiments on rat liver ribosomes using different experimental techniques (Huvos *et al.*, 1970).

In an effort to use the ribonuclease digestion on ribosomes as an approach for analyzing ribosomal function, we attempted to measure the three biological activities of these digested ribosomes. We would like to know if nuclease digestion preferentially impairs one of the three activities. Our results, at the present, do not allow us to make such a distinction mainly because of the relatively large margin of error (15–20%) in the two binding assays (Figures 2 and 3). However, our data do indicate that both the binding of AA-tRNA and mRNA are impaired by nuclease digestion.

As a first step in the attempt to correlate the functions of the ribosomes and the structure of these RNA regions, we have examined the elution profile of the RNA fragments remaining in the 70S ribosomal particle after RNase T_1 treatment. Relatively few fragments were obtained even after considerable treatment with RNase T_1. This observation is in agreement with those of Ehresmann and Ebel (1970) and Santer and Szekely (1971) after RNase T_1 digestion under conditions which would lead to total hydrolysis of the free RNAs.

Finally our data showed that some of the RNA fragments remaining in the ribosomes after enzymatic digestion possessed nonphosphorylated 3′-hydroxyl terminus and thus could be oxidized with sodium periodate followed by reduction with sodium borohydride. These fragments must have been generated from the original 3′-hydroxyl termini of the tRNA molecules, because the action of RNase T_1 should yield 3′-phosphorylated guanylic acid which would not undergo such oxidation–reduction reaction. In addition these termini have been shown to be mainly uridine and thus could not have been produced by RNase T_1. Hence our data indicated that the terminal oligonucleotides of the rRNA molecules lie within the protected area. Our findings that uridine and adenosine were the termini is in agreement with those on the intact 5S, 16S, and 23S rRNA of *E. coli* (McIlreavy and Midgley, 1967; Brownlee *et al.*, 1967).

References

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brownlee, G. G., Sanger, F., and Barrell, B. G. (1967), *Nature (London) 215*, 735.

Cohn, W. E., and Volkin, E. (1951), *Nature (London) 167*, 483.

Cox, R. A. (1969), *Biochem. J. 114*, 753.

DeWachter, R., and Fiers, W. (1967), *J. Mol. Biol. 30*, 507.

Ehrenstein, G. (1967), *Methods Enzymol. 12*, 588.

Ehresmann, C., and Ebel, J. P. (1970), *Eur. J. Biochem. 13*, 577.

Fellner, P., Ehresmann, C., Ebel, J. P., and Blasi, O. (1970), *Eur. J. Biochem. 13*, 583.

Fenwick, M. L. (1968), *Biochem. J. 107*, 481.

Gesteland, R. F. (1964), *J. Mol. Biol. 8*, 496.

Godson, G. N., and Cox, R. A. (1970), *Biochim. Biophys. Acta 204*, 489.

Huvos, P., Vereczkey, L., and Gaal, O. (1970), *Biochem. Biophys. Res. Commun. 41*, 1020.

Lee, J. C. (1971), *Biochim. Biophys. Acta 235*, 435.

Lee, J. C., and Gilham, P. T. (1966), *J. Amer. Chem. Soc. 88*, 5685.

Lee, J. C., and Ingram, V. M. (1969), *J. Mol. Biol. 41*, 431.

Lee, J. C., and Quintanilla, I. V. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 889.

Lee, J. C., Weith, H. L., and Gilham, P. T. (1970), *Biochemistry 9*, 113.

Leppla, S. H., Bjoraker, B., and Bock, R. M. (1968), *Methods Enzymol. 12B*, 236.

McIlreavy, D. J., and Midgley, J. E. M. (1967), *Biochim. Biophys. Acta 142*, 47.

McPhie, P., and Gratzer, W. B. (1966), *Biochemistry 5*, 1310.

Möller, W., Amons, R., Groene, J. C. L., Garett, R. A., and Terhorst, C. P. (1969), *Biochim. Biophys. Acta 190*, 381.

Moore, P. B. (1966), *J. Mol. Biol. 22*, 145.

Nirenberg, M. W. (1963), *Methods Enzymol. 6*, 17.

Nirenberg, M. W., and Leder, P. (1964), *Science 145*, 1399.

Nomura, M. (1970), *Bacteriol. Rev. 34*, 228.

RajBhandary, U. L. (1968), *J. Biol. Chem. 243*, 556.

Salas, M., Smith, M. A., Stanley, W. M., Jr., Wahba, A. J., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3988.

Santer, M. (1963), *Science 141*, 1049.

Santer, M., and Szekely, M. (1971), *Biochemistry 10*, 1841.

Sarker, P. K., Yung, J. T., and Doty, P. (1967), *Biopolymers 5*, 1.

Spirin, A. S., and Gavrilova, L. P. (1969), The Ribosomes, New York, N. Y., Springer-Verlag, p 40.

Levallorphan-Induced Accumulation of ppGpp in *Escherichia coli*†

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ABSTRACT: Levallorphan, a structural analog of morphine, was found to induce the accumulation of guanosine 5′-diphosphate, 3′-, or 2′-diphosphate in both a relaxed and a stringent strain of *Escherichia coli*.

Starvation for a required amino acid or restriction of the aminoacylation of tRNA causes a severe reduction of RNA accumulation in stringent (*rel*$^+$) but not relaxed (*rel*) strains of *Escherichia coli* (Edlin and Broda, 1968). Several seconds prior to the onset of this so-called stringent response, an unusual nucleotide, guanosine 5′-diphosphate, 3′-, or 2′-di-

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