Nuclease Action on *Escherichia coli* Ribosomes and Its Application to Sequence Studies on Ribosomal Ribonucleic Acid*

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ABSTRACT: The rRNA contained in 70S, 50S, and 30S ribosomes of *Escherichia coli* is sensitive to the action of pancreatic and T_1 RNase.

At very low enzyme levels some sites are hydrolyzed generating large RNA fragments. As the enzyme level is raised the number of nuclease sensitive sites increases until a limit is reached. At this ratio of enzyme to substrate (1:20–1:40) there is produced a series of RNA fragments whose band pattern in gel electrophoresis experiments cannot be altered by additional enzyme or longer incubation periods. This reveals that there are many protected areas in the intact ribosome which are resistant to nuclease treatment and are not found in free rRNA. Three RNA fragments, obtained from T_1 RNase-treated labeled 30S ribosomes, were completely digested with T_1 RNase and "fingerprinted." They appear to be relatively pure components with the smallest composed of about 60 nucleotides. These RNA pieces appear suitable for sequence work.

Recent work of Traub and Nomura (1969) on reconstitution of 30S and 50S ribosomes from their constituent RNA and protein components has proven that sufficient information for the assembly of ribosomal particles is contained in the primary structure of the interacting molecules. Investigation of the primary structure of rRNA and of the way it attaches to proteins to form ribosomal particles has therefore acquired special interest. The primary structure of 16S RNA of *Escherichia coli* ribosomes has been studied by Fellner and Sanger (1968), who determined sequences around the methylated bases in both 16S and 23S RNA, and by Fellner *et al.* (1970a), who established the structure of nucleotides present in a complete RNase T_1 digest of isolated 16S RNA. In the present paper another approach to this problem is described, namely, a method for digesting RNA within 70S, 50S, and 30S particles. This method provides RNA fragments suitable for nucleotide sequence studies and at the same time may give information on some structural features of the ribosomal particle by discriminating between exposed and protected areas of the RNA in ribosomes.

Nuclease degradation of RNA within ribosomes has been observed by several authors (Santer, 1963; Santer and Smith, 1966; Székely *et al.*, 1967; Fenwick, 1968; Möller *et al.*, 1969; Cox, 1969; Godson and Cox, 1970; Ehresmann and Ebel, 1970; Hüvös *et al.*, 1970). Pancreatic RNase was shown to remove varying amounts of RNA from 50S and 30S ribosomes of *E. coli* without altering the sedimentation properties of ribosomes. Under defined conditions, however, there was an upper limit to the RNA that could be removed from these particles (Santer and Smith, 1966). While these results showed that part of the rRNA is strongly protected against RNase action, the results of Fenwick (1968), as well as of Hüvös *et al.* (1970), showed that, in the case of animal cell ribosomes, some sites of the RNA in the particles were readily accessible to low levels of pancreatic RNase, resembling mRNA in their RNase sensitivity. It may thus be supposed that the easily accessible sites are at the surface of the particle while the protected areas found by Santer and Smith (1966) are in the interior of the ribosome or, if at the surface, are attached to protein. It seemed therefore of interest to determine what kinds of RNA molecules remain in 30S, 50S, and 70S ribosomes treated with nuclease under different conditions. The aim of such a study would be to try to generate large fragments of rRNA which represent protected areas and which would be suitable for nucleotide sequence analysis.

While this work was being completed, Fellner *et al.* (1970b) reported on a similar approach to sequencing long fragments of 16S RNA obtained by digesting the 30S ribosomal subunit of *E. coli* with RNase T_1 and also free sRNA (Ehresmann *et al.*, 1970).

Materials and Methods

Ribosomes. In most experiments, including all those using T_1 RNase, ribosomes of *E. coli* MRE 600 were used; a few early experiments used ribosomes of *E. coli* CA 265. Nonradioactive ribosomes were prepared according to Nirenberg (1963). Dissociation into subunits was achieved by dialysis against 0.01 M Tris buffer (pH 7.6) containing 0.02 M KCl and 10^{-4} M magnesium acetate. ^{32}P-Labeled ribosomes were prepared from *E. coli* MRE 600 cells grown in a low-phosphate medium in the presence of [^{32}P]P_i. ^{32}P-Labeled cells were lysed by freezing and thawing in the presence of lysozyme followed by treatment with sodium deoxycholate, according to Ron *et al.* (1966). Ribosomes were sedimented from a 26,000*g* supernatant at 130,000*g*. Dissociation into subunits was achieved by passing the ribosome suspension through a Sephadex G-200 column in 0.01 M Tris (pH 7.6)–0.02 M KCl–10^{-4} M magnesium acetate. Ribosomal subunits were separated by sucrose density gradient centrifugation, using a 5–

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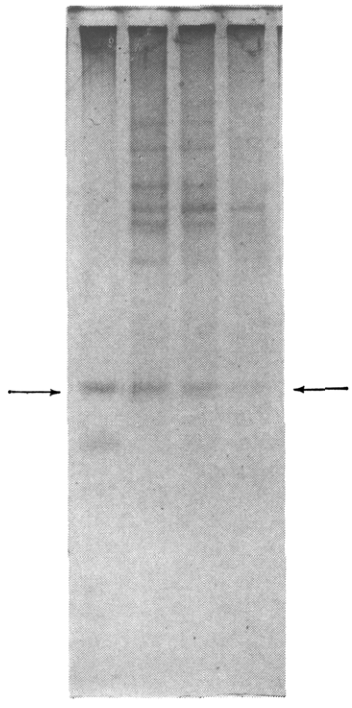

FIGURE 1: Polyacrylamide gel pattern of RNA derived from 70S ribosomes treated with different levels of pancreatic RNase. Each incubation mixture contained 2.2 mg of ribosomes (1.4 mg of RNA) in 0.01 Tris buffer (pH 7.8), 0.06 M KCl, 0.01 M magnesium acetate, and 0.006 M 2-mercaptoethanol in a total volume of 100 μl. Incubation temperature, 0° for 30 min. RNA extracted two times with phenol at 60° in the presence of 1% sodium dodecyl sulfate and 0.01 M EDTA. RNA was precipitated with ethanol in the presence of 4 M guanidinium chloride. The gel contained 5% acrylamide and 0.25% bisacrylamide in 0.04 M Tris (pH 8.2) and 0.001 M EDTA. RNA was disolved in Tris buffer with 10–20% autoclaved sucrose for better layering of sample on gel. The RNA bands were stained with methylene blue. From left to right: control (RNA from undigested ribosomes) and RNA from ribosomes digested with 2, 0.5, and 0.1 μg of RNase, respectively. Arrows indicate position of 5s RNA.

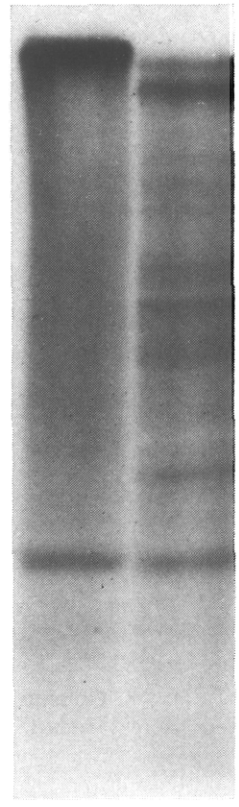

FIGURE 2: Polyacrylamide gel pattern of RNA derived from ^{32}P-labeled 70S ribosomes treated with pancreatic RNase. Ribosomes (10 μg) and pancreatic RNase (0.01 μg) incubated in 0.1 M Tris buffer (pH 8.2), 0.04 M KCl, and 0.01 magnesium acetate in a total volume of 26 μl. Incubation at 23° for 50 min. RNA obtained in the same way as in Figure 1. Gel electrophoresis carried out as in Figure 1. RNA bands visualized by radioautography. Left: 0-min digestion; right: 50-min digestion.

20% gradient in the above buffer. Subunits were located by counting aliquots of fractions and were recovered by raising the Mg^{2+} concentration of pooled fractions to 10^{-3} M and centrifuging at 130,000g for 20 hr. Nuclease digestion of 70S ribosomes was performed in the presence of 10^{-2} M magnesium acetate. Dissociated subunits were usually treated in buffer containing 10^{-3} M magnesium acetate. The volume of the digestion mixture was 20–100 μl.

RNA fragments were isolated by extracting the digested ribosomes three times with phenol at room temperature, followed by precipitation with ethanol. In some cases hot phenol extraction (60°) in the presence of 1% sodium dodecyl sulfate and 0.01 M EDTA was applied. The precipitated RNA was washed with ethanol.

[^{32}P]RNA bands were separated on flat polyacrylamide gels according to the method of Adams *et al.* (1969). Radioautography to locate RNA bands was also carried out according to procedures described by these authors. The gel contained 10% polyacrylamide and 0.5% bisacrylamide in 0.04 M Tris (pH 8.4) with 7 M urea when a high concentration of enzyme was used and 5% acrylamide–0.25% bisacrylamide–0.001 EDTA when low enzyme levels were used. Disc polyacrylamide gels were run according to Richards *et al.* (1965). Two-dimensional fractionation of oligonucleotides and nucleotides, "fingerprinting," and sequence techniques were those of Sanger and associates (1965, 1968). Enzite RNase was purchased from Miles-Seravac, Ltd., and was used after washing several times with 0.1 M Tris (pH 8.2) containing 0.01 M $MgCl_2$. It was suspended in the same buffer and columns were made up in the capillary of a Pasteur pipet, the extent of digestion being controlled by the length of the column and the rate at which the suspension of ribosomes was passed through the column.

T_1 RNase (EC 2.7.7.26) was purchased from Calbiochem. Pancreatic RNase (EC 2.7.7.16) was obtained from Worthington Biochemical Corp.

Results

Mild Degradation of 70S Ribosomes with Pancreatic RNase. Ribosomes were treated under different conditions with increasing amounts of pancreatic RNase. The RNA fragments produced were isolated from the particles and separated by gel electrophoresis.

Figure 1 shows the gel pattern of the RNA obtained from 70S ribosomes which were treated with varying concentrations of enzyme in the cold (enzyme:RNA ratios from 1:14000 to 1:700). Figure 2 is the gel pattern of the RNA obtained from ribosomes labeled with ^{32}P and treated with a slightly higher level of enzyme at 23°; in both experiments a 5% gel has been used. It is possible to obtain some idea of the size range of the molecules produced under the various conditions because the 5S RNA emerges intact and is clearly visible in all preparations, running slightly behind the bromophenol blue dye marker. It is clear that as the enzyme level is raised and the

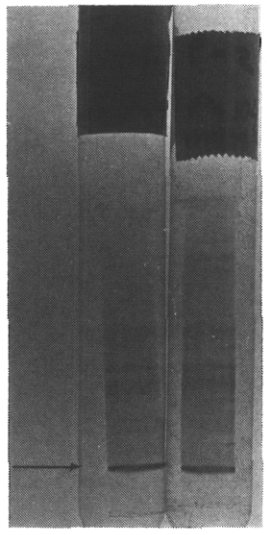

FIGURE 3: Polyacrylamide gel pattern of RNA obtained from 30S ribosomes treated with high levels of pancreatic RNase. Ribosomes (4.9 mg) in 0.01 M Tris buffer (pH 7.8), 0.01 M KCl, and 0.0001 M magnesium aceate were treated with 70 μg of enzyme at 23° (tube at left) and at 0° (right) for 30 min. Purified RNA was dissolved in 0.1 M magnesium acetate buffer (pH 5.6) and electrophoresced in 7.5% gel according to the procedure of Richards *et al.* (1965). Arrow indicates bromophenol blue dye marker.

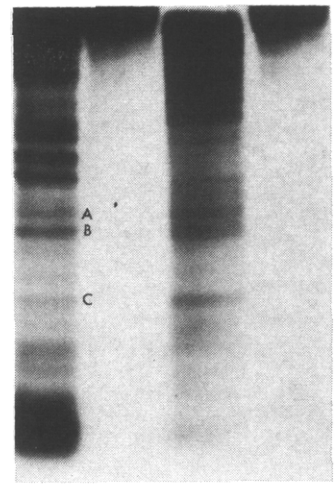

FIGURE 4: Radioautograph of polyacrylamide gel electrophoretic separation of RNA obtained from T_1 RNase-treated 50S and 30S ribosomes. Separate solutions of ^{32}P-labeled 50 and 30 S in 0.01 M Tris (pH 7.8), 0.01 M KCl, and 0.001 M magnesium acetate were treated at 23° for 45 min with 1 μg of enzyme/OD_{260} unit. RNA was electrophoresced in flat gels (Adams *et al.*, 1969) using 10% acrylamide–0.5% bisacrylamide in 0.04 M Tris (pH 8.4) containing 7 M urea. From left to right: RNA from T_1 RNase-treated 30S ribosomes, 30S control, RNA from RNase T_1-treated 50S ribosomes, 50S control. Control preparations indicate slight breakdown of RNA.

temperature of incubation raised, more RNA bands are seen in the gels, demonstrating that more breaks are made in the RNA of the ribosome. At an enzyme-to-substrate level of 1:700 almost all of the rRNA can enter the 5% gel. Most of the bands are larger than 5 S. Under similar conditions, isolated rRNA is degraded to small fragments.

Stronger degradation was obtained with larger amounts of an insoluble form of RNase A bound to CM-cellulose (Enzite RNase). A solution of 70S ribosomes in 0.1 M Tris (pH 8.2)–0.01 M $MgCl_2$ was passed through a 9 × 0.2 cm column of Enzite RNase at room temperature for 10 min. The RNA purified from these ribosomes yielded a number of bands in 10% polyacrylamide gels, but only part of it did enter the gel. At low temperature and one-fifth the amount of insoluble enzyme, little degradation of the RNA in 70S ribosomes occurred but free rRNA was substantially degraded, even with columns 0.7 cm long.

Strong Degradation of 30S Ribosomes with Pancreatic RNase. A different picture emerges after treatment of these particles with high levels of enzyme under different environmental conditions. Figure 3 illustrates the polyacrylamide gel pattern of RNA obtained from 30S ribosomes treated with pancreatic RNase in a weight ratio of 1:40 (enzyme:RNA), at 10^{-4} M Mg^{2+} ion concentration and at two different temperatures. In this experiment the gel concentration is 7.5%. At 23° all the RNA molecules obtained from the 30S ribosomes are clustered in the front 50% of the gel, if the bromophenol blue marker is considered the front. The average *s* value of these bands is about 5. At 0° many more RNA fragments are visible in the top 50% of the gel. These fragments are considerably larger than the ones obtained at 23° but at the same time, at 0°, there still appear to be many of the bands observed at the higher temperature. The other important fact is that, although we are using a higher gel concentration than in the experiments reported in Figures 1 and 2, all the RNA recovered from these 30S ribosomes enters the gel, even the RNA obtained from ribosomes incubated at 0°.

Digestion of Ribosomes with RNase T_1. 70S ribosomes are more resistant to RNase T_1 than to pancreatic RNase. Even when applying a 1:40 enzyme to substrate ratio, only a few rather large fragments are obtained. The effect of RNase T_1 on ribosomal subunits is also different from that of pancreatic RNase.

Figure 4 shows the radioautograph of an electrophoretogram of RNA obtained from ^{32}P-labeled 50S and 30S ribo-

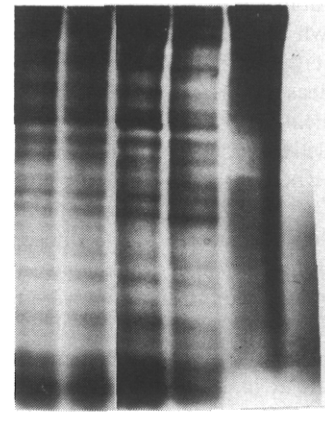

FIGURE 5: Radioautograph of polyacrylamide gel electrophoretic separation of RNA obtained from ^{32}P-labeled 30S ribosomes treated under different conditions with T_1 RNase. ^{32}P-Labeled 30S ribosomes used in Figure 6 were treated as follows, from left to right: 2.0, 1.0, and 0.5 μg of enzyme per optical density unit for 45 min at 23°. Column 4 contains RNA from ribosomes treated with 1.0 μg/optical density unit for 3 hr at 23°, and column 5 is a control, which was incubated for 3 hr at 23°. There is some "self-digestion" yielding some large bands in the gels. The positions occupied by these bands are not similar to the experimental RNA. The control also shows some streaking of the RNA down the wall of the glass enclosing the gel.

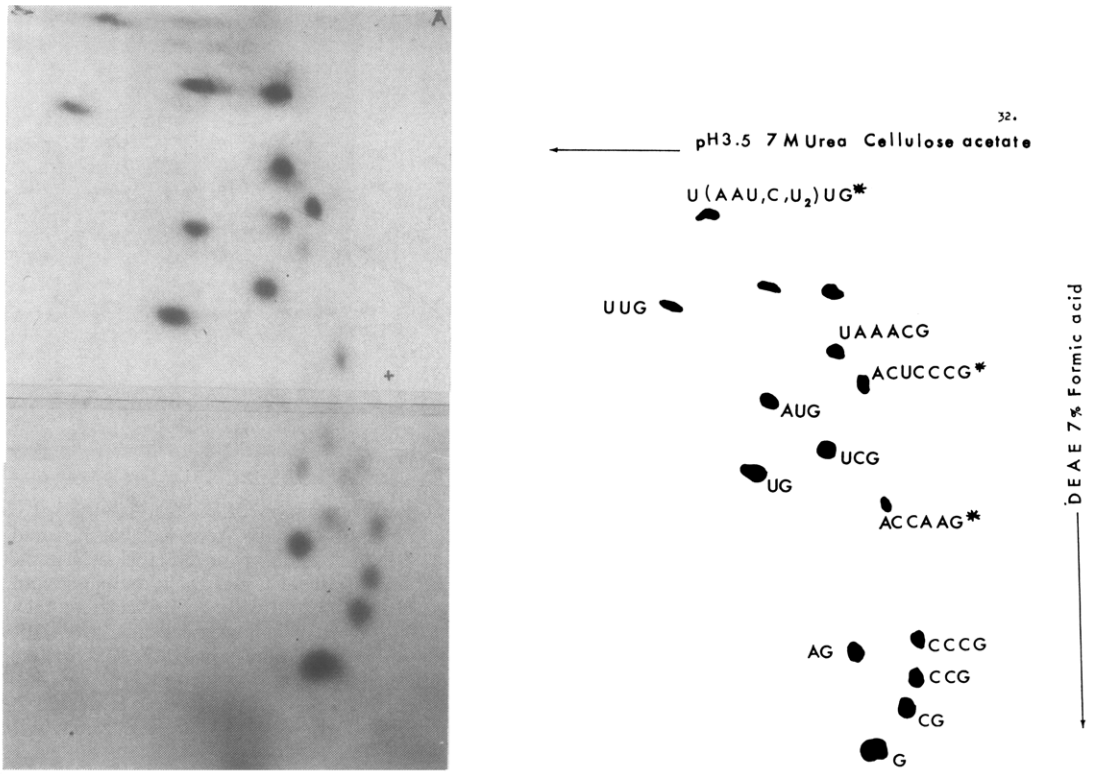

FIGURE 6: Radioautograph of two-dimensional electrophoretic separation of complete T_1 RNase digest of band A. The RNA fragment was obtained from gels according to the methods of Adams *et al.* (1969) and digested and "fingerprinted" according to the procedures of Sanger *et al.* The + shows position of the blue marker. Structure of nucleotides established by the techniques of Sanger and associates. *Nucleotides identified by comparing degradation products with structure of nucleotides described by Fellner *et al.* (1970a). Bands A, B, and C were eluted from different gels at different times; their relative positions are indicated in Figure 4.

somes which were treated separately with RNase T_1 at an enzyme to substrate ratio of 1:40. A large number of RNA fragments are visible in the gel, but a considerable amount of RNA has not entered the 10% gel. The controls show little degradation of the RNA in 30S and 50S particles. The difference in sensitivity toward the two enzymes is in accord with unpublished experiments in which one of us (M. Santer) had shown that RNase T_1 removes less than 10% of the RNA from either subunit under conditions where pancreatic RNase releases more than 20% of the RNA.

In the experiments described thus far, different conditions of digestion yielded different RNA patterns. It was important, however, to test whether there is a limit to the amount of degradation of the rRNA, a limit which might be imposed by the three-dimensional structure of the ribosome. To test this possibility, the following experiment was carried out. 30S ^{32}P-labeled ribosomes used in Figure 4 were treated with different levels of T_1 RNase for one fixed time period and a separate aliquot was treated with a 1:40 enzyme to substrate ratio for 3 hr. These data are presented in Figure 5. All the RNA gel patterns are essentially identical. Thus, an increase in the enzyme to substrate ratio from 1:80 to 1:20 or even 1:10 (not shown), or extending the incubation period by a factor of 4, does not appear to alter the band pattern. It can thus be concluded that there is a limit to RNase T_1 attack on the ribosome.

The reproducibility of the band pattern suggested that this technique may be useful for producing well-defined RNA fragments for sequence studies. The question to be answered then is whether these bands represent pure fragments with a unique sequence or are they mixtures of molecules? Three different bands, for simplicity labeled A, B, C, were obtained from the gel by the procedures of Adams *et al.* (1969), completely digested with T_1 RNase, and fingerprinted by the methods devised by Sanger and associates. Figures 6–8 contain the radioautographs of the fingerprints of these three bands. Included in each figure is a diagram with the sequence or the nucleotide composition of many of the spots.

It appears that bands A and B contain two identical hexanucleotides, ACCAAG and UAAACG (although the former is faint on the fingerprint of band A), and also a longer nucleotide which on pancreatic RNase treatment yields the following products: 3U, AAU, C, and G. This spot may be identical with that described by Fellner *et al.* (1970a), as nucleotide 3, which is U(AAU,C,U_2)UG. These similarities of known nucleotide fragments and the similar position of two other unidentified spots indicate that band B overlaps with the larger RNA molecule called band A. Band C, which has a completely different fingerprint pattern than bands A and B, is a smaller molecule than the other two. It has three characteristic larger nucleotide fragments, one of whose sequences has been determined. It is AACCUG, which is nucleotide 67 in the Fellner *et al.* (1970a) paper. This sequence was deduced from the following data. On complete pancreatic RNase hydrolysis the fragment yielded AAC, 2C, U, and G; after carbodiimide addition and pancreatic RNase hydrolysis: UG, 2C, and AAC. Snake venom digestion established AAC as the 5′ end of the molecule.

The "simple" nature of these fingerprint patterns and the estimated number of nucleotides, based on comparison of the mobility of these bands to that of *E. coli* tRNA, indicate that they are relatively pure.

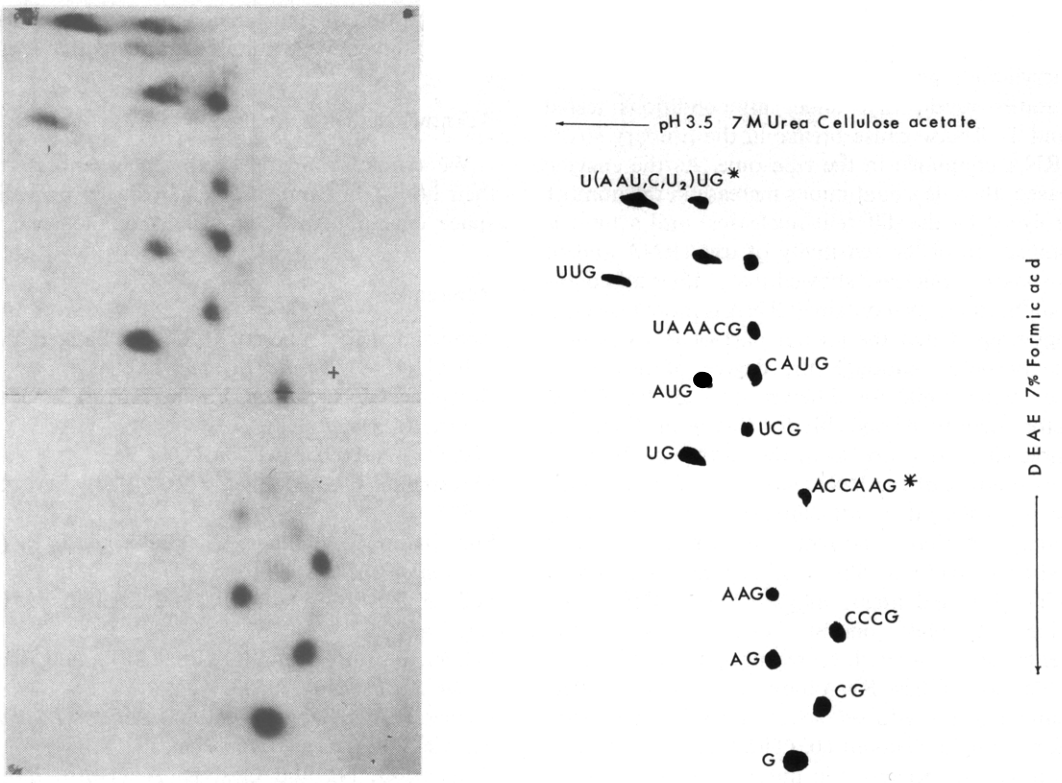

FIGURE 7: Radioautograph of the two-dimensional electrophoretic separation of complete T_1 RNase digest of band B. Band B obtained and treated as described in Figure 6.

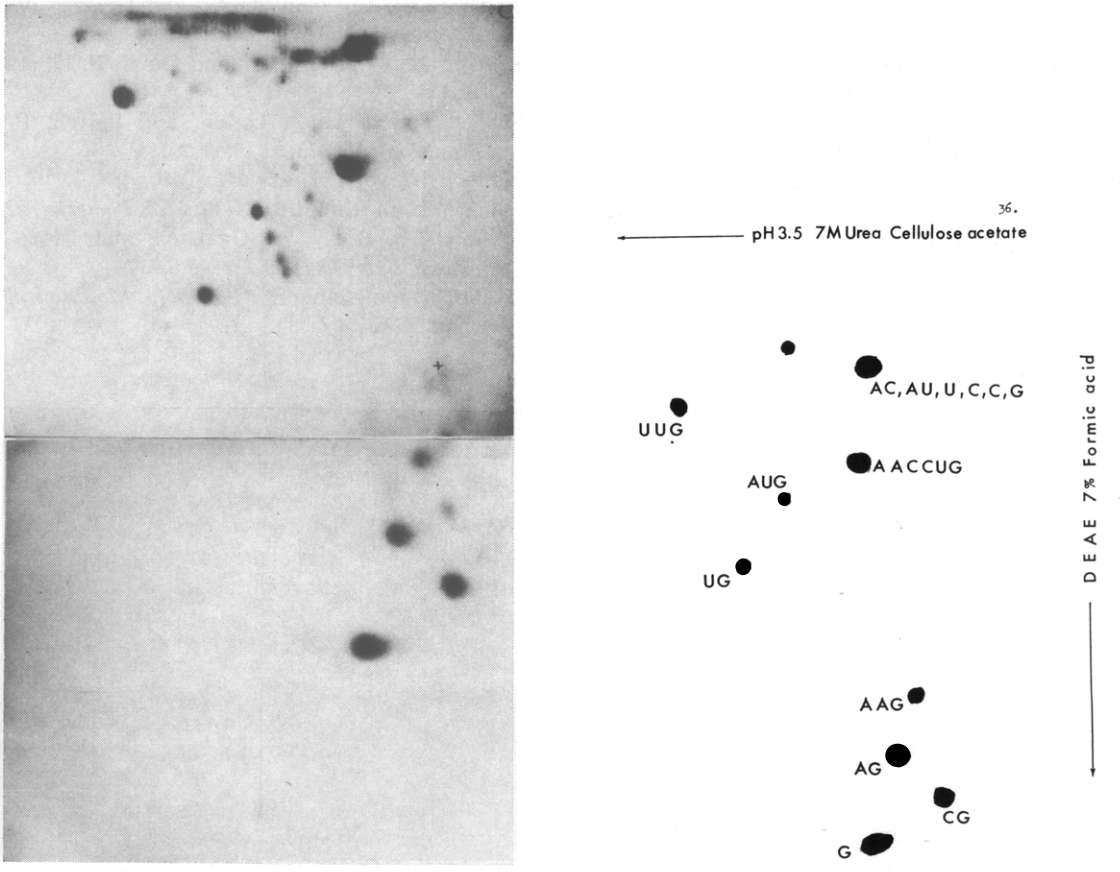

FIGURE 8: Radioautograph of the two-dimensional electrophoretic separation of the complete T_1 RNase digest of band C. Band C obtained and treated as described in Figure 6.

Discussion

The polyacrylamide gel patterns obtained from nuclease-treated ribosomes reveal that under all conditions tested pancreatic and T_1 RNase cause breaks in the primary structure of the RNA contained in the ribosome. As the enzyme level is increased, there is a continuous increase in the number of sites hydrolyzed by the different nucleases until a limit is reached. Comparison of the sensitivity of free rRNA and of RNA in ribosomes to nuclease showed that, under all conditions of digestion, ribosome-contained RNA is more resistant. These findings suggest that the greater part of RNA is protected in the particles, although the degree of protection varies between wide limits for different sites of the RNA. Different factors may be responsible for this protection. The easily accessible sites are probably at the surface of the ribosome, whereas the rest of the RNA molecule may be buried in the inside or protected by attachment to proteins or by strong secondary structure. The fact that there is a definite limit to digestion of RNA in ribosomal subunits proves that there are fully protected areas, inaccessible to RNase T_1. After digestion with high concentrations of this enzyme, RNA fragments can be isolated, representing these fully protected areas. The size of these RNA molecules varies considerably. The number of nucleotides observed on the fingerprint of band C, for example, is about 60. (The exact number is at the moment unknown because the number of G, AG, and CG residues has not been quantitated.) Since the molecule migrates slightly behind the bromophenol blue marker in gels, which is near the bottom of the picture, it is clear that there are many larger molecules than C, including bands A and B. In fact, near the top of the gel the RNA molecules are approximately 100–150 nucleotides long. There are even larger molecules than that, which do not enter the gel and are seen at the origin. The fact that a limit digest with RNase T_1 generates RNA fragments varying greatly in size does not support a "symmetrical" structure for ribosomal particles (Cox, 1969). Finally, it is important to stress that protected fragments isolated from 30S ribosomes exhaustively digested with RNase T_1 give reasonably simple fingerprint patterns. A rough comparison between the number of nucleotides estimated from fingerprint patterns and the mobility of the band in polyacrylamide gels relative to *E. coli* tRNA marker suggests that these bands are relatively pure. Thus, the technique of maximal digestion of ribosomal subunits with RNase T_1 produces RNA fragments very suitable for nucleotide sequence studies.

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