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The Specific Cleavage of Ribonucleic Acid from Reticulocyte Ribosomal Subunits*

Hannah Gould

ABSTRACT: Ribonucleic acid (RNA) isolated from each of the two subunits of reticulocyte ribosomes has been digested with pancreatic ribonuclease. The conditions were adjusted so that the early stages of digestion could be observed by separation of the products by polyacrylamide gel electrophoresis. The course of digestion is highly specific suggesting that certain regions are exceptionally prone to enzymatic attack. The patterns of degradation of the two types of RNA are entirely different implying that these regions are distributed at characteristic intervals in each case. The first products of digestion are relatively large, having molecular weights in the range of 250,000–400,000. As the digestion proceeds, the higher molecular weight species are degraded into smaller fragments in the range of 20,000–250,000 molecular weight.

The results indicate that the number of vulnerable regions is of the order of 5 for the 30S component and 3 for the 19S component. It is suggested that these regions are attacked preferentially because the RNA chain is folded in a specific manner which leaves them uniquely exposed.

Excellent resolution of high molecular weight ribonucleic acid (RNA) species in the range of 2–14 S has been obtained using polyacrylamide gel electrophoresis (Richards *et al.*, 1965; McPhie *et al.*, 1966). This technique has been used in the present study to analyze the early stages in the enzymatic digestion of ribosomal RNA from rabbit reticulocyte subunits.

Previous studies have shown that specific intermediates may be formed upon mild nuclease digestion of total ascites ribosomal RNA (*e.g.*, Huppert and Pelmont, 1962). Studies of the RNA from the two subunits, individually, have so far been confined to the examination of the products formed upon the complete digestion with specific nucleases. Aronson (1963), and Sanger *et al.* (1965), have shown that the complete enzymatic digests contain different relative amounts of various oligonucleotides, and therefore the nucleotide sequences are different.

Likewise, the results to be described below indicate that the molecular weights and numbers of products formed in the early stages of digestion of RNA from the two subunits differ. It is inferred that the regions most susceptible to enzymatic attack are distributed in a

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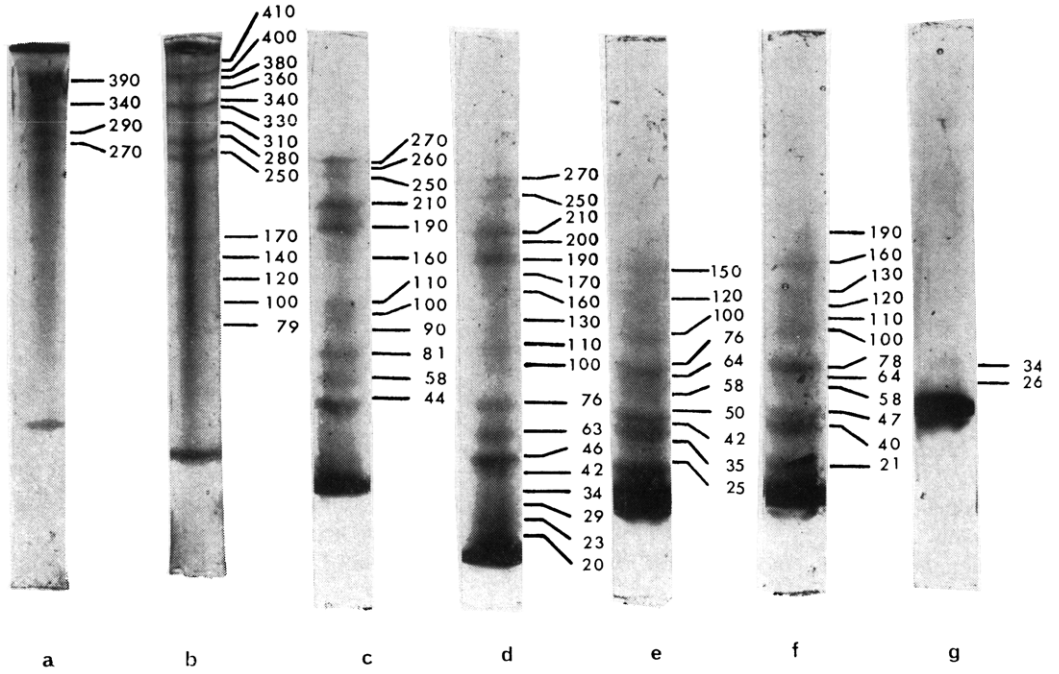

FIGURE 1: Digestion of whole ribosomal RNA from rabbit reticulocytes. Each sample contained 400 μg of RNA in 0.25 ml of 0.1 M Tris–HCl buffer, pH 7.6. (a) Sample incubated in the absence of enzyme for 22 hr at 20°; (b) 1×10^{-2} μg of ribonuclease for 30 min at 20°; (c) 1 μg of enzyme for 5 min at 0°; (d) 1 μg of enzyme for 5 min at 20°; (e) 1 μg of enzyme for 30 min at 20°; (f) 1 μg of enzyme for 5 min at 37°; (g) 1 μg of enzyme for 30 min at 37°. After incubation the samples were treated as described in the Experimental Section. The numbers to the right of the photographs are the calculated molecular weights $\times 10^{-3}$ of the resolved fragments.

characteristic manner in each case. The results demonstrate in addition that the degradation of ribosomal RNA may be finely controlled, and that relatively large fragments containing overlapping sequences may be obtained which should be useful in the analysis of primary structure.

Experimental Section

Preparation of Reticulocyte Ribosomes and Ribosomal Subunits. Rabbits weighing 2–3 kg were made anemic by five consecutive daily injections of 0.8 ml of neutralized 2.5% phenylhydrazine, and were bled on the seventh day by cardiac puncture. The reticulocytes were washed and lysed, and the ribosomes isolated from the lysate and fractionated by zone centrifugation in sucrose gradients in the manner previously described (Arnstein *et al.*, 1964, 1965). Ribosomes were dissociated into subunits with EDTA, and the subunits were fractionated by zone centrifugation as described previously (Gould *et al.*, 1966).

Isolation of RNA. Whole RNA was prepared from polysomes, and 30S and 19S RNA were isolated from the corresponding subunits after concentration from sucrose gradient fractions (Gould *et al.*, 1966), by using guanidinium chloride to dissociate the ribonucleoprotein, and ethanol to precipitate the RNA (Cox, 1965). The RNA was extracted with water and stored frozen at −10°. RNA from unfractionated ribosomes gave two peaks in the analytical ultracentrifuge with $s^0_{20,w} = 30$ S and 19 S, in the ratio of 2.8:1, respectively. RNA isolated from the larger subunit and from the smaller subunit gave single peaks in the analytical ultracentrifuge with sedimentation coefficients of 30 S and 19 S, respectively.

Digestion of RNA with Pancreatic Ribonuclease. Aliquots of a stock solution of bovine pancreatic ribonuclease (C. F. Boehringer and Soehne) at either 150 μg/ml or 1.5 μg/ml were added to the RNA solution (2 mg/ml in 0.1 M Tris buffer, pH 7.6) to give the desired enzyme–substrate ratio, generally about $^1/_{400}$ or $^1/_{40,000}$ by weight. Samples were incubated in a thermoregulated water bath at the required temperature, and were then plunged into ice water. One-tenth volume of bentonite at 10 mg/ml in water was added immediately to absorb ribonuclease and was removed by centrifugation. The RNA digest was concentrated by precipitating the RNA with 0.1 volume of 1 M sodium acetate and 2 volumes of ethanol, centrifuging the precipitate at 3000 rpm in the MSE Mistral centrifuge for 15 min, and redissolving the pellet in a minimum quantity of water to give a concentration of approximately 10 mg/ml.

Electrophoresis Experiments. Acrylamide gels (5% BDH Cyanogum 41, 0.25 M Tris–HCl buffer, pH 8.5, 0.1% dimethylaminoethyl cyanide, and 0.1% ammonium

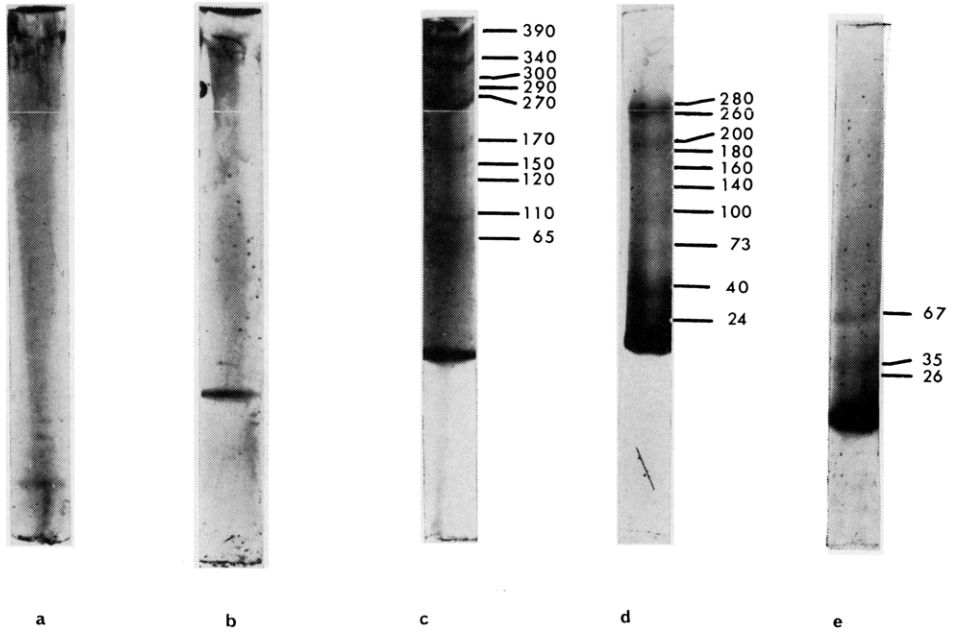

FIGURE 2: Digestion of RNA from the larger subunit of rabbit reticulocyte ribosomes. The conditions are the same as for the digestion of whole ribosomal RNA described in the caption to Figure 1. (a) Sample before incubation with enzyme; (b) 1×10^{-2} μg of enzyme for 5 min at 20°; (c) 1 μg of enzyme for 5 min at 20°; (d) 1 μg of enzyme for 30 min at 20°; (e) 1 μg of enzyme for 1 hr at 20°.

persulfate) were prepared as described in the preceding paper (McPhie *et al.*, 1966). A sufficient number of Whatman 3MM paper disks impregnated with the RNA solution were placed on top of the gels. Electrophoresis was carried out using the same buffer system and running conditions as McPhie *et al.* (1966). The gels were extruded into dye solution (1% acridine orange, 2% lanthanum acetate, in 15% acetic acid), and after about 24 hr the gels were destained using the apparatus described by Richards *et al.* (1965). The gels were stored in 15% acetic acid at 4°.

Photography of Acrylamide Gels. The gels were placed in a perspex container and covered over with water. The container was placed on a glass plate suspended over a white card, illuminated from the sides. The camera was placed above the gels and photographs were taken using high contrast ortho-Ilford G552 film and a yellow filter. Films were developed with Johnson's Universal 1 + 7 for 2.5 min at 68°F.

Analysis of Electrophoresis Patterns. It has been shown by McPhie *et al.* (1966) that a linear relationship exists between the R_F value of a given RNA species (R_F = distance travelled by the RNA/distance travelled by the solvent front) and its sedimentation coefficient. We have used the equation $MW = (6.68 - 5.63\ R_F)^2 \times 10^4$ (see McPhie *et al.*, 1966) to obtain the molecular weights of the RNA in the zones seen in the photographs of the polyacrylamide gels which are presented below.

The relative amounts of RNA in various zones were determined by densitometry of the polyacrylamide gels. A Joyce Loebl microdensitometer was used to record the optical density pattern. The ratio of the area under a given peak to the total area was used as a measure of the relative concentration of the RNA species.

Results

Degradation of Whole Reticulocyte Ribosomal RNA. A graded series of specific degradation products of whole reticulocyte ribosomal RNA may be obtained with pancreatic ribonuclease by varying the amount of enzyme, or the length, or temperature of incubation, as shown in the first figure. An early stage in the degradation of whole RNA may be seen in Figure 1b. In this experiment 400 μg of RNA was exposed to 1×10^{-2} μg of ribonuclease for 30 min at 22°. The most prominent zones are found near the origin of the sample at a distance corresponding to the molecular weight range of about 250,000–400,000.

Three later stages were observed when the amount of enzyme was increased to 1 μg. When the samples were incubated for 5 min at 0° (Figure 1c) or 22° (Figure 1d), the major zones were found in the molecular weight range of 200,000–250,000 and 60,000–90,000. When the samples were incubated for 30 min at 22° (Figure 1e) or 5 min at 37° (Figure 1f), the major zones were found in the region of the gel corresponding to molecular weights below 100,000. Finally, treatment for 30 min at 37° resulted in degrading all the material to molecular weight less than 50,000 (Figure 1g).

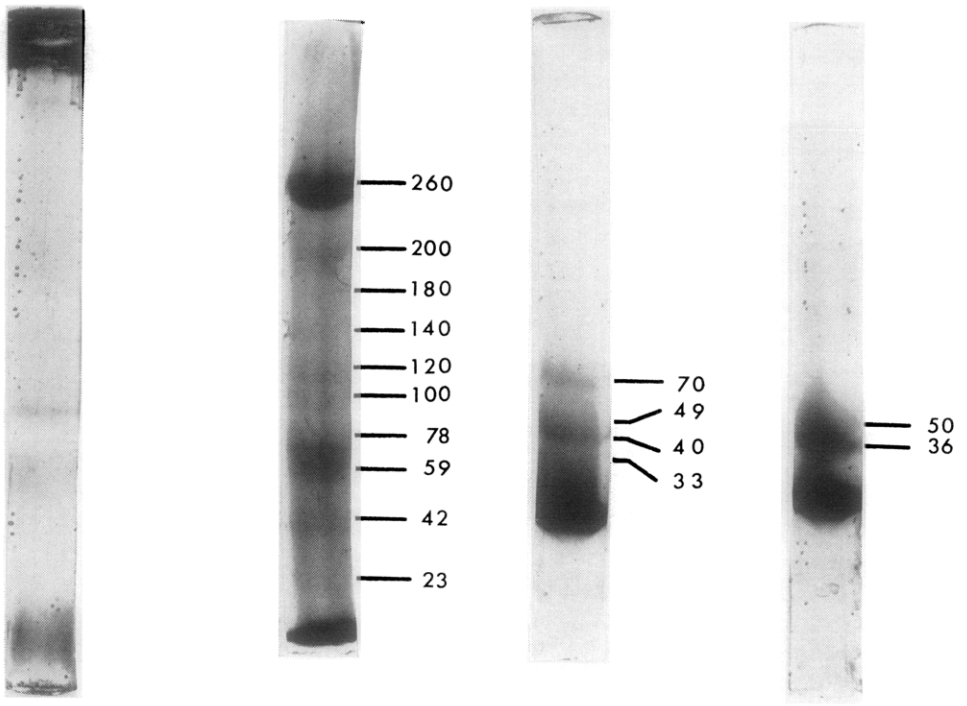

FIGURE 3: Digestion of RNA from the smaller subunit of rabbit reticuloyte ribosomes. The conditions are the same as for the digestion of whole ribosomal RNA described in the caption to Figure 1. (a) Sample before incubation with enzyme; (b) 1 μg of enzyme for 5 min at 0°; (c) 1 μg of enzyme for 5 min at 20°; (d) 1 μg of enzyme for 30 min at 20°.

It is of interest that the incubation of whole RNA for 24 hr at 22° in the absence of enzyme caused scarcely any degradation to molecular weights of less than 400,000 (Figure 1a). Hence there is no doubt that the original RNA was effectively free from contaminating nucleases and that the electrophoretic patterns b–g may be attributed to pancreatic ribonuclease digestion.

Degradation of 30S RNA. The degradation of whole ribosomal RNA reflects mainly that of the 30S component which comprises 70% of the total. Thus, treatment of 30S RNA (Figure 2) produces fragments which give electrophoresis patterns closely similar to that of the unfractionated material.

Mild treatment with ribonuclease (400 μg of RNA, 1×10^{-2} μg of ribonuclease, 5 min at 22°) is not sufficient to degrade 30S RNA to fragments which are small enough (below 400,000 molecular weight) to enter the gel, but some digestion is indicated by the appearance of low molecular weight material (below 20,000 molecular weight) which travels with the solvent front (Figure 2b, *cf.* undigested 30S RNA in Figure 2a).

Longer incubation (*e.g.*, 30 min) at the same enzyme concentration produced the array of fragments seen in Figure 2c (*cf.* Figure 1b), having molecular weights from 100,000 upwards. At the higher enzyme level (400 μg of RNA, 1 μg of ribonuclease, 5-min incubation at 22°), the pattern reveals the shift to lower molecular weights, less than 100,000 (Figure 2e; *cf.* Figure 1e and f). With longer incubations (*e.g.*, 30 min), only the smallest fragments still remained (Figure 3e; *cf.* Figure 1d).

Degradation of 19S RNA. When 400 μg of 19S RNA was degraded with 1 μg of ribonuclease at 0° for 5 min, a very intense band corresponding to a molecular weight of 260,000 was observed in the electrophoresis pattern (Figure 3b). Microdensitometry of the gel revealed that this zone contained about 16% of the total RNA. Faint zones corresponding to lower molecular weight species can also be seen. It is apparent from Figure 3c (5-min incubation at 22°) and Figure 3d (30 min at 22°) that these species increase in amount as the digestion proceeds.

Discussion

It is remarkable that the electrophoresis patterns of ribonuclease-digested ribosomal RNA, from rabbit reticulocytes, no less than from yeast (McPhie *et al.*, 1966), exhibit sharp rather than diffuse zones, and it is of interest to consider the possible implications of this finding. The relation $Z = (n + 1)(n + 2)/2$, described in the previous paper (McPhie *et al.*, 1966), may be used to calculate the number of breaks, n, which may be deduced from the appearance of a given number, Z, of

zones in the acrylamide gels. In this study about 20 distinct species were observed in the degradation of RNA from the larger subunit and about 10 in the degradation of RNA from the smaller subunit. The number of regions attacked by the enzyme may therefore be set at a minimum of 5 and 3, respectively. This is an exceedingly small number in view of the very large size of the RNA molecules involved.

The high degree of specificity of the ribonuclease attack might be explained in several ways. Ribonuclease attacks principally the 5′-phosphodiesterase linkages adjacent to pyrimidine residues. Since, however, both components of reticulocyte ribosomal RNA are rich in pyrimidines (Gould *et al.*, 1966), the specificity of the enzyme cannot alone account for the results. Even if ribonuclease attack were restricted to the unfolded regions of the RNA chains, the number of pyrimidine residues is still too large (Cox, 1962; Gould and Cox, unpublished observations). Clustering of pyrimidine residues could bring about the production of a small number of large fragments after treatment with ribonuclease, but the results of both Aronson (1963) and Sanger *et al.* (1965) argue against this explanation. The specificity of ribonuclease for the sequence about pyrimidine residues (Witzel and Barnard, 1962) might account for the results. However, the facts which are known about the nearest neighbor frequencies in mammalian ribosomal RNA (J. R. Tata, personal communication) do not lend support to this explanation.

A more plausible model envisages the rate of hydrolysis to be limited mainly by the presence of secondary and/or tertiary structure in ribosomal RNA. About 70% of residues are involved in base pairing, or secondary structure. Moreover, there is fairly good evidence that the helical regions in both chains are relatively short, involving only of the order of 4–17 nucleotides each on average (Cox, 1966; Cox and Gould, unpublished results; Timasheff *et al.*, 1961). The number of separate amorphous regions connecting the helices must therefore be of the same order, and since they contain less than half as many residues they must be correspondingly shorter on the average. Heterogeneity in the size of the unfolded regions could account for the specificity of ribonuclease attack, since long flexible regions might be expected to be more vulnerable than short rigid ones. Tertiary structure, for instance, the alignment of helical segments for which there is some evidence in ribosomes (Spirin, 1964; Langridge, 1963), could also presumably determine the course of the digestion.

The digestion of ribosomal RNA with pancreatic ribonuclease is further notable in that relatively large fragments appear first, and these are progressively degraded to lower molecular weight species. This implies that the primary sites of enzymatic attack are rather widely spaced and the secondary sites of attack are within the initial fragments. Either then there are initially present sites which are labile, but less so than those of the first cleavage, or new sites become available in consequence of the first set of breaks.

It is of interest to examine the electrophoretic patterns for evidence of the presence of subunit structure within the RNA chains. In the case of the digestion of RNA from the larger ribosomal subunit, a pattern emerges whereby the fragments appear to form a series decreasing approximately by 10,000 or multiples of 10,000 in molecular weight.

In the case of RNA from the smaller subunit the largest, and very prominent, fragment observed had a molecular weight of 250,000 and comprised around 16% of the total material. The molecular weight of an undegraded 19S mammalian RNA is approximately 600,000 (Hamilton, 1964). The fragment therefore represents about one-half of the original molecule. It will be of interest to determine whether two species of identical size are produced by specific cleavage at the center of the RNA chain, or whether this fragment represents a section out of the middle which is released by the attack of separate regions on either side. It may be possible to resolve this question by attempting to observe yet earlier stages in the digestion of the 19S component.

In the early stages of the digestion of both types of RNA the appearance of high molecular weight species near the origin was accompanied by a densely staining solvent front which was absent in the control (*e.g.*, Figure 2a,b). This finding supports the view that specific regions, perhaps those with poorly developed secondary structure, rather than bases in a particular sequence, are the primary targets of the enzyme.

Finally it may be pointed out that the technique of vertical acrylamide gel electrophoresis affords an unrivalled means of analyzing the structure of high molecular weight RNA such as ribosomal RNA, since the resolved fragments can be excised from the gel (McPhie *et al.*, 1966) and used for further work.

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Multiple Electrophoretic Zones Arising from Protein–Buffer Interaction*

John R. Cann

ABSTRACT: Moving-boundary experiments demonstrate that in the pH range 6.2–9.2, BSA (bovine serum albumin) interacts reversibly with phosphate–borate and borate buffers to give electrophoretic patterns showing two or more peaks. The two or three zones shown on cellulose acetate by BSA in these buffers arise from the same interaction and are not indicative of true heterogeneity.

In the many applications of zone electrophoresis to biological problems, it is imperative that cognizance be taken of the fact that multiple zones need not necessarily indicate heterogeneity.

Recently Cann and Goad, (1965a,b) have presented a theory of electrophoresis of reversibly interacting systems of the type

$$P + nHA \rightleftharpoons P(HA)_n \qquad (1)$$

where P represents a protein molecule or other macromolecular ion in solution and $P(HA)_n$ its complex formed by binding n moles of a small, uncharged constituent, HA, of the solvent medium, *e.g.*, undissociated buffer acid. It is assumed that P and $P(HA)_n$ possess different electrophoretic mobilities and that equilibrium is established instantaneously. One of the results of computations for zone electrophoresis (Cann and Goad, 1965b) is that under appropriate conditions a single macromolecule, interacting with an uncharged constituent of the solvent, can give two zones. Since zone electrophoresis is such a powerful method for separating macromolecules and has found extensive application to a variety of biological problems, it is important to verify the prediction experimentally. The experiments described herein were designed with this in mind and demonstrate that a protein can give multiple zones due to interaction with the buffer solvent.

Experimental Section

Moving-boundary electrophoresis was carried out in the standard 11-cc Tiselius cell with the Spinco Model H electrophoresis–diffusion instrument. Schlieren patterns were recorded photographically with the cylindrical lens system. Values of the mobilities, $cm^2\ sec^{-1}\ v^{-1} \times (10^{-5})$, and apparent mobilities are shown above or beside the corresponding peaks in the patterns presented in Figure 1.

Zone electrophoresis was carried out on 5 × 20-cm S and S cellulose acetate strips with a Colab Shandon apparatus. Between 10 and 15 μl of protein solution was applied to the strip with a Beckman sample applicator. On completion of electrophoresis the zone patterns were developed in the usual manner by staining with Ponceau S. In the case of fractionation experiments in phosphate–borate buffer, the zones on an unstained strip were visualized with ultraviolet light. After cutting out the desired zone, the rest of the strip was stained to make certain that there was no contamination with the other zone. The protein was eluted by floating the excised zone on 70 μl of buffer in a capped vial for 3–4 hr with occasional agitation. In the case of borate buffer, a narrow longitudinal piece was cut from one

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