The Effect of Pancreatic Ribonuclease on Rabbit Reticulocyte Ribosomes and its Interpretation in Terms of Ribosome Structure

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1. Parts of the 16s and 30s RNA species of reticulocytes are readily hydrolysed by pancreatic ribonuclease. The biological activity of the ribosomes is diminished after treatment with low concentrations of the enzyme (e.g. 1 ng. of ribonuclease/2.5 mg. of polyribosome fraction/ml.). A high proportion of the chain scissions are 'hidden' owing to the secondary structure of the RNA moiety. 2. As the concentration of ribonuclease is increased RNA is lost from the ribosome. About 20–30% of the RNA may be removed from the ribosome without altering appreciably its sedimentation coefficient or its appearance in the electron microscope. 3. The amount of RNA removed from the ribosome is not increased by raising the concentration of enzyme from about 1 μ g. to 2.5 mg. of ribonuclease/2.5 mg. of polyribosome fraction/ml., or by increasing the temperature from 0° to 30°, or by first converting the RNA moiety into a single-stranded form before exposure to ribonuclease. 4. Untreated polyribosomes aggregate at about 75°, whereas ribosomes treated with ribonuclease aggregate at about 45°. The aggregates that are found on heating ribosomes after enzymic hydrolysis contain about 40–50% of the complement of RNA of intact ribosomes. 5. From the size of the fragments of RNA isolated from RNA-depleted ribosomes it is inferred that there is one site/60–100 nucleotides that is sensitive to ribonuclease. 6. The RNA moiety of RNA-depleted ribosomes has some double-helical character as shown by the optical properties and X-ray-diffraction pattern of ribonuclease-treated ribosomes and by the 'melting' properties of the isolated RNA. 7. Subparticles prepared by titration with an excess of EDTA are readily hydrolysed by ribonuclease to fragments of $S_{20,w}$ less than 4s, in contrast with the intact particle.

Ribosomes are ribonucleoprotein particles of about 3.4×10^6 daltons that are composed of 40–60% RNA (for reviews see Arnstein, 1963; Petermann, 1964; Spirin, 1967). The RNA moiety appears to be freely accessible to small reagents such as formaldehyde (see Cox, 1969) and cationic dyes (see, e.g., Cotter, McPhie & Gratzer, 1967). The reaction of larger molecules with the RNA moiety is of interest in studying the distribution of RNA between the surface and interior of the ribosome. Pancreatic ribonuclease (13 683 daltons, about 32 Å in diameter) was considered to be suitable in view of its size and because the sensitive sites can be readily detected by measuring the size of the RNA moiety after the protein is dissociated. A study of the effect of pancreatic ribonuclease on rabbit reticulocyte ribosomes and on subparticles produced by titration with EDTA was therefore undertaken. The principal results are that subparticles produced by EDTA treatment are readily hydrolysed to slowly sedimenting material (about 5s or less). Some of the sensitive regions are masked

in the intact ribosomes and native subparticles since they appear to survive exposure at 0–30° to massive doses of enzyme. However, the RNA moiety is attacked even by low concentrations of ribonuclease, leading to 'hidden' breaks and to the loss of biological activity. Higher concentrations of enzyme leads to the production of fragments of $S_{20,w}$ 4s on average (after 'hidden' breaks are revealed), suggesting that for RNA from the larger subparticle there are about 40 sensitive sites separated by about 105 residues on average.

EXPERIMENTAL

Chemicals. Guanidinium chloride (about 6M) was prepared by neutralizing A.R. guanidinium carbonate with conc. HCl as described by Cox (1966). For a 4M solution $E_{280} < 0.05$. A.R. formaldehyde was used without further purification. All other reagents were of A.R. quality. Pancreatic ribonuclease was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Sodium dodecyl sulphate, obtained from British Drug Houses Ltd., Poole, Dorset, was recrystallized from aqueous ethanol.

Ribosomal preparations. Reticulocytes were obtained from rabbits that had been given daily intraperitoneal injections of neutralized 2.5% (w/v) phenylhydrazine hydrochloride (0.3 ml./kg. body wt.) for 6 or 7 days; the cells were lysed in 5 mM-MgCl₂ (Schweet, Lamfrom & Allen, 1958). The ribosomes and polyribosome fractions were isolated by differential centrifuging (Arnstein, Cox, Gould & Potter, 1965).

Isolation of RNA. Ribosomal RNA was isolated by precipitation as the guanidinium salt (Cox, 1966).

Determination of protein. The protein content of ribosomes was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Spectrophotometry. The u.v.-absorption spectra and extinctions were measured with a Unicam SP.700 spectrophotometer. The sample-cell holder was an electrically heated copper block that could be maintained at any temperature in the range 25–95° to better than 0.1°. The observed extinctions measured at temperatures other than 20° were corrected for changes in concentrations due to the thermal expansion of water.

Ultracentrifuging. MSE low-speed (Major, Mistral and 13) centrifuges, MSE high-speed (40 and Super-Speed 50) ultracentrifuges and the Spinco model L ultracentrifuge were used for all preparative work. The high-speed ultracentrifuges were used for zone centrifuging.

Sucrose gradients were prepared by layering 30%, 25%, 20% and 15% (w/v) sucrose solutions in the appropriate buffers. Fractions were collected with a siphon.

A Spinco model E ultracentrifuge fitted with u.v.-absorption optics was used for analytical work. In the optical path the camera was replaced by a photoelectric cell that scanned the slit, and the movement of the sedimenting species was followed by means of an X-Y recorder. Cells of 12 mm. path length were used in all cases. All analytical studies were made at about 20° with ribosomes (50 mg./l.) or RNA (25 mg./l.).

Optical rotation. Optical rotatory dispersions over the range 220–500 nm. were measured with a Bellingham and Stanley Polarmatic recording spectropolarimeter. The instrument was calibrated with standard sucrose solutions. The extinction at 260 nm. was never more than 1.7 and the measured rotations were directly proportional to concentration. Readings were independent of slit width (1 nm.).

Polyacrylamide-gel electrophoresis. The method used was described by Richards, Coll & Gratzer (1965). Polyacrylamide gel (10%, w/v) was prepared by mixing 1 vol. of 50% (w/v) Cyanogum 41 (obtained from British Drug Houses Ltd.) in 0.486 M-tris-0.1 M-HCl buffer, pH 8.47, with 0.1 vol. of dimethylaminoethyl cyanide and 0.1 vol. of 10% (w/v) ammonium persulphate. Portions (2.5 ml.) of the gel were transferred to glass tubes (9 mm. diam.) sealed at one end with Parafilm, held vertically and left overnight to polymerize. RNA [0.25 mg. in 0.25 ml. of 5% (w/v) sucrose] was applied to the gel, layered with 3.3 mM-tris-0.03 M-barbituric acid, pH 7.0, which also served as the medium for electrophoresis (300 V, 30 mA), which was continued for about 1 hr. The gels were removed, left overnight in 1% (w/v) Acridine Orange-2% (w/v) lanthanum acetate in 15% (v/v) acetic acid. The unbound dye was later removed by repeated washing with 10% (v/v) acetic acid.

Measurement of the buoyant density of ribosomes. The method described by Perry & Kelley (1966) was used. The

ribosome pellets were suspended in buffer (2 ml. of 0.24 M-KCl-5 mM-MgCl₂-50 mM-triethanolamine, pH 7.8) and dialysed overnight against the same buffer. Neutralized formalin (40%, w/v) was added to give 8% solution. The excess of formaldehyde was removed by dialysis for 24 hr. against buffer. A gradient of CsCl was prepared by running 2.5 ml. of CsCl (ρ 1.700, 9.333 g. added to 7.666 ml. of buffer) against 1.0 g. of CsCl plus 1 ml. of buffer, plus 1 ml. of ribonuclease-treated solution (E_{260} approx. 10) plus 0.25 ml. of ¹⁴C-labelled ribosomes (E_{260} approx. 3.0) to give a linear gradient from ρ 1.4 to ρ 1.7. Fractions were collected after centrifuging for 16 hr. at 35 000 rev./min. (Spinco SW 39 rotor) at about 4°. The tube was punctured and six drops were collected in each tube. Groups of three fractions were diluted to 1 ml., and E_{260} and E_{280} were then measured. Ribosomes were then precipitated with 10% (w/v) trichloroacetic acid, washed with 5% (w/v) trichloroacetic acid, then with ethanol-ether and finally with ether on to Whatman GF 82 filters, and counted in a toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazol-2-yl)benzene mixture with a Beckman 200 scintillation counter. The refractive index of every fourth fraction was measured, from which the density was calculated.

The weight fraction, x , of RNA was calculated from the equation:

$$x = \frac{\bar{v}_{\text{protein}} - \bar{v}_{\text{obs.}}}{\bar{v}_{\text{protein}} - \bar{v}_{\text{RNA}}}$$

where $\bar{v}_{\text{protein}}$, $\bar{v}_{\text{RNA}}$ and $\bar{v}_{\text{obs.}}$ are respectively the partial specific volumes of ribosomal protein, ribosomal RNA and the observed value for ribosomes. Perry & Kelley (1966) use the values $\bar{v}_{\text{RNA}}$ 0.526 and $\bar{v}_{\text{protein}}$ 0.800.

¹⁴C-labelled ribosomal RNA. Anaemic rabbits were injected with 0.2 mc of [6-¹⁴C]orotic acid (specific radioactivity 60.8 mc/m-mole; obtained from The Radiochemical Centre, Amersham, Bucks.) 24 hr. before they were killed. About 2% of the uracil residues of reticulocyte ribosomal RNA were labelled. The introduction of 2% of labelled uracil residues has negligible effect on the buoyant density of the ribosomes.

Base analyses of RNA. The nucleotide composition of RNA was obtained by the method of Katz & Comb (1963). RNA was hydrolysed with 0.4 M-KOH for 72 hr. at 35°. The solution was concentrated by rotary evaporation and the residue was neutralized with 2 M-HCl. The solution was passed through a column of Dowex 50 (H⁺ form). The sample was loaded with 50 mM-HCl and eluted first with 50 mM-HCl to yield UMP, then with water to elute first GMP and then AMP together with CMP. The mixture of AMP and CMP was passed through a column of Dowex 1 (formate form) that had first been washed with 50 mM-formic acid to elute CMP, and then with 0.4 M-formic acid to elute AMP. The appropriate fractions were pooled and evaporated to dryness, each residue was dissolved in 5 ml. of 1 mM-MgCl₂-10 mM-potassium phosphate buffer, pH 7.5, and the concentration of nucleotide was measured spectrophotometrically.

RESULTS

Effect of ribonuclease on the molecular weight of EDTA-subparticles. The polyribosome fraction in 0.25 M-sucrose-25 mM-potassium chloride-1 mM-

Fig. 1. Sedimentation profile of EDTA-subparticles before and after treatment with ribonuclease. The polyribosome fraction in 0.25M-sucrose-25mM-KCl-1mM-MgCl₂-50mM-tris-HCl buffer, pH 7.6, was made 50mM with respect to EDTA, pH 7.6 (cf. Gould, Arnstein & Cox, 1966). Samples (1ml.) of the solution (2.7mg. of ribonucleoprotein/ml.) were treated with ribonuclease for 30min. at 20° and layered on to 15-30% (w/v) sucrose gradients (in 10mM-KCl-1.5mM-MgCl₂-10mM-tris-HCl buffer, pH 7.6) and centrifuged for 16hr. at 17500 rev./min. (Spinco SW 25 rotor). (a) Control (no added ribonuclease); (b) 8 ng. of ribonuclease/2.7 mg. of ribonucleoprotein/ml.; (c) 80 ng. of ribonuclease/2.7 mg. of ribonucleoprotein/ml.

magnesium chloride-50mM-tris-hydrochloric acid, pH 7.6, was made 50mM with respect to EDTA, pH 7.6. The final concentration was 2.7mg. of polyribosome fraction/ml. Samples (1ml.) were treated with 0, 8 and 80ng. of ribonuclease for 30min. at about 20°, layered on to 15-30% (w/v) sucrose gradients and centrifuged for 16hr. at 17500 rev./min. (Spinco SW 25 rotor). Treatment with 8ng. of ribonuclease (3ng./mg. of ribosomes) affected the sedimentation rate of the smaller but not the larger ribosomal subparticle (see Fig. 1b). When the concentration of ribonuclease was increased from 8ng. (3ng./mg. of ribosomes) to 80ng. (30ng./mg. of ribosomes) the subparticles were hydrolysed to fragments that scarcely moved from the meniscus. The fragments had the same u.v.-absorption spectrum as intact subparticles, showing that the RNA/protein ratio was unchanged. Thus EDTA-subparticles appear to be as sensitive to hydrolysis by ribonuclease as isolated ribosomal RNA (cf. Cox & Kanagalingam, 1967).

Effect of enzymic hydrolysis on the properties of ribosomes. (a) Biological activity of the ribosome fraction. The ability of ribosomes to incorporate labelled amino acids into acid-insoluble protein

was affected by treatment with ribonuclease. A typical experiment is summarized in Table 1, which shows the effect of enzymic hydrolysis on both the endogenous activity and on the polyuridylic acid-directed synthesis of polyphenylalanine. The addition of 0.75ng. of ribonuclease (corresponding to 6ng./mg. of RNA) to each assay tube decreased the incorporation of [¹⁴C]phenylalanine into acid-insoluble protein from 2.6nc to 0.8nc/tube. The ability of the ribosome fraction to synthesize protein was decreased after preincubation with the same concentration of ribonuclease before the other components of the cell-free system were added. After 60min. pretreatment only 0.15nc/tube was found insoluble in acid. The ability of ribosome fraction to respond to polyuridylic acid was also impaired: in the absence of added ribonuclease 20.6nc/tube was incorporated into protein compared with 13.7nc/tube when ribonuclease was added to the complete system, and compared with 6.25nc/tube obtained after the ribosome fraction was preincubated with ribonuclease for 60min. As reported earlier (Arnstein, 1961) ribosomes preincubated with 60ng. of ribonuclease/mg. of RNA have little or no biological activity as measured in

Table 1. *Effect of ribonuclease treatment (10 ng./2.5 mg. of polyribosome fraction/ml.) at 20° on the activity of ribosomes in protein biosynthesis*

Ribonuclease (0.05 ml. of a solution containing 0.1 μ g. of ribonuclease/ml.) was added to samples (0.5 ml.) of the stock polyribosome solution (2.5 mg./ml.) at 20° for periods of 0–60 min. before the sample was assayed in the cell-free system. The solutions 3–16 inclusive had the same composition and differed only in the period for which the ribosomes were pretreated with ribonuclease. No attempt was made to remove the added ribonuclease. Each assay tube contained (in 0.5 ml.) ribosomes (0.25 mg.), pH 5 enzyme fraction (1 mg. of protein), $MgCl_2$ (2.5 μ moles), KCl (25 μ moles), tris-HCl, pH 7.6 (12.5 μ moles), GSH (5 μ moles), sucrose (85 μ moles), an energy-generating system (ATP, GTP, potassium phosphoenolpyruvate and pyruvate kinase) and amino acids including [^{14}C]-phenylalanine (50 μ C/ μ mole). The reaction was stopped after incubation for 1 hr. at 37°, the precipitate formed on the addition of trichloroacetic acid was filtered on Oxoid membranes and the radioactivity was measured at infinite thinness with a low-background (1.5 c.p.m.) ultra-thin-Micromil-window gas-flow Geiger counter (model 181-B; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) giving a counting efficiency of approx. 5%.

Tube no.	Pretreatment of ribosomes	Addition to standard assay	Radioactivity of acid-insoluble protein (nc)
1	None	None	2.6
2	None	50 μ g. of polyuridylic acid	20.6
3	None	1 ng. of ribonuclease	0.8
4	None	1 ng. of ribonuclease, 50 μ g. of polyuridylic acid	13.7
5	2 min. exposure to ribonuclease	None	0.7
6	2 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	13.1
7	4 min. exposure to ribonuclease	None	0.65
8	4 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	11.8
9	8 min. exposure to ribonuclease	None	0.5
10	8 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	9.7
11	16 min. exposure to ribonuclease	None	0.3
12	16 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	9.3
13	32 min. exposure to ribonuclease	None	0.2
14	32 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	6.9
15	60 min. exposure to ribonuclease	None	0.15
16	60 min. exposure to ribonuclease	50 μ g. of polyuridylic acid	6.25
17	Ribosomes omitted	None	Nil
18	Ribosomes omitted	50 μ g. of polyuridylic acid	Nil

our standard assay either in the presence or in the absence of polyuridylic acid. The design of the experiment makes it unlikely that the decrease in the polyuridylic acid-directed biosynthesis of acid-insoluble protein was due solely to residual enzyme degrading the polynucleotide since the composition of the solutions assayed was the same in each case (including traces of ribonuclease). The ribosomes differed only in the length of the pretreatment with ribonuclease.

(b) Sedimentation profile of ribosomes after treatment with low concentrations of ribonuclease. Samples (1 ml.) of the polyribosome fraction (2.7 mg./ml.) were treated with 0, 2.7 and 13.5 ng. of ribonuclease for 30 min. at about 20°, then layered on to 15–30% (w/v) sucrose gradients and centrifuged for 3 hr. at 25 000 rev./min. (MSE SW25 rotor). Treatment with 2.7 ng. of ribonuclease (1 ng./mg. of ribosomes) was sufficient to destroy the polyribosome pattern (Fig. 2).

(c) Sedimentation properties of EDTA-subparticles produced from ribonuclease-treated ribosomes. Samples (1 ml.) of the polyribosome fraction

(2.7 mg./ml.) were first treated with 0, 13.5 and 81 ng. of ribonuclease for 30 min. at about 20° and were then made 50 mM with respect to EDTA, pH 7.6. The larger subparticle tended to persist after this treatment, but the smaller subparticle and possibly part of the larger subparticle fraction was hydrolysed to slow-sedimenting species that scarcely left the meniscus. The presence of a fraction sedimenting as the larger subparticle after treatment with 30 ng. of ribonuclease/mg. of ribosomes is in contrast with the sedimentation patterns found (cf. Figs. 1c and 3c) when subparticles were first produced before treatment with ribonuclease. When the polyribosome fraction was treated with 3 μ g. of ribonuclease/mg. of ribosomes before the addition of EDTA, pH 7.6, no subparticles were detected on zone centrifuging (see Fig. 3d).

(d) Molecular size of the RNA moiety of ribosomes after hydrolysis with 60 ng. of ribonuclease/mg. of RNA moiety. Polyribosomes (50 mg.) were dissolved in 0.25 M-sucrose–25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris-hydrochloric

Fig. 2. Sedimentation profile of polyribosomes after treatment with low concentrations of ribonuclease. Samples (1 ml.) of the polyribosome fraction (2.7 mg./ml.) were treated with ribonuclease, layered on to 15–30% (w/v) sucrose gradients (in 10 mM-KCl–1.5 mM-MgCl₂–10 mM-tris-HCl buffer, pH 7.6) and centrifuged for 3 hr. at 25000 rev./min. (MSE SW25 rotor). (a) Control (no added ribonuclease); (b) 2.7 ng. of ribonuclease/2.7 mg. of polyribosomes/ml.; (c) 13.5 ng. of ribonuclease/2.7 mg. of polyribosomes/ml.

acid, pH 7.6 (5 ml.), and 1.5 μ g. of ribonuclease (0.3 μ g./10 mg. of polyribosomes/ml.; 60 ng./mg. of RNA moiety) was added at 22°. Samples were removed after 0, 10, 20, 40, 80, 120 and 240 min. and immediately added to 2 vol. of 6 M-guanidinium chloride at 0°, a solvent in which ribonuclease is inactive (Sela, Anfinsen & Harrington, 1957). RNA was precipitated by the addition of ethanol (0.5 vol.) at 0°, and was purified by dissolution in and reprecipitation from 4 M-guanidinium chloride and then dissolved in 10 mM-potassium chloride. The sedimentation profile of a dilute solution (about 30 μ g./ml.) of each RNA sample was measured before and after secondary structure was destroyed to reveal 'hidden breaks' (for discussion see Cox & Kanagalingam, 1967). The most convenient procedure was to prepare two dilutions of each sample that had the same final composition but differed in the order in which the reagents were added. Since titration to low pH values is sufficient to break down the double-helical structure of ribosomal RNA the procedure followed was as follows. The control was prepared by first neutralizing 1 mM-

hydrochloric acid with potassium phosphate buffer to make the solution 10 mM with respect to phosphate buffer, pH 7.5, and RNA was then added. In this case RNA was kept at pH 7 throughout. A second dilution was prepared by adding RNA to 1 mM-hydrochloric acid and the solution was then neutralized with phosphate buffer. In this case the RNA was first adjusted to pH 3 before the solution was neutralized. The solutions were kept at 0–20°, the RNA was kept in acid for less than 2 min. and bentonite (1 mg./ml.) was present throughout.

The sample that was kept at pH 7 throughout was found (Fig. 4) to have a sedimentation profile comparable with, but with broader boundaries than, that of RNA from untreated ribosomes, and some material was found to sediment slower than the 17s component, as described by Cox & Arnstein (1963). The amount of 10–12s material increased with time.

The sample that was exposed to low pH values was found to have a different sedimentation profile, since the average sedimentation coefficient, $S_{20,w}$, was about 9s after incubation for 10 min. and about

Fig. 3. Sedimentation properties of the product of EDTA treatment of polyribosomes previously exposed to ribonuclease. The materials and conditions were described in Fig. 1. In this case ribonuclease was added to the polyribosome fraction before EDTA. (a) Control (no added ribonuclease); (b) 13.5 ng. of ribonuclease/2.7 mg. of polyribosomes/ml. (the sedimentation profile of the ribosome fraction before the addition of EDTA is given in Fig. 2c); (c) 81 ng. of ribonuclease/2.7 mg. of polyribosomes/ml.; (d) 12 μ g. of ribonuclease/4 mg. of polyribosomes/ml.

7 s after incubation for 240 min. (Fig. 4). In contrast, the sedimentation profile of RNA from the control ribosomes was scarcely affected by the acid treatment.

A similar result was obtained when the molecular size of the RNA samples was measured by electrophoresis in 10% polyacrylamide gels. The RNA samples that were kept at pH 7 throughout failed to penetrate the gels, although a faint band was noted close to the origin after 10 min. treatment or less; about five slow-moving bands were noted after hydrolysis for 240 min. In contrast, the samples that were treated to reveal hidden breaks formed about 15 bands or more (see Fig. 5).

Some effects of extensive treatment with ribonuclease on the properties of ribosomes. The polyribosome fraction (2.5 mg. of ribosomes/ml.) was divided into six samples (1 ml.) to which 0, 0.1, 1, 10, 75 and 750 μ g. of ribonuclease were added respectively. The samples were left at 20° for 15 min., then layered on to 15–30% (w/v) sucrose gradients and centrifuged for 3.5 hr. at 25 000 rev./min. (MSE SW 25 rotor). A well-defined ribosome fraction was found together with u.v.-absorbing material that remained at the meniscus (Fig. 6). The spectrum of the slow-sedimenting species was close to that of RNA and

the amount of protein determined by the Lowry method was not more than 10%. The fraction of the extinction at 260 nm. that remained at the meniscus from this and a similar experiment carried out at 0° is given as a function of added ribonuclease in Fig. 7, which shows that no more u.v.-absorbing material was released from the ribosome on increasing the amount of ribonuclease from 10 μ g. to 750 μ g. A similar result was obtained when the release of [14 C]uracil from labelled ribosomes was measured. Thus increasing the concentration of ribonuclease did not destroy the ribosome at 20°. There is a limit to the amount of ribonuclease that can be added because eventually the ribosomes are precipitated (see Fig. 7). The precipitate is redissolved on the addition of more ribosomes. The same amount of cytochrome *c*, another basic protein of about the same molecular weight, also precipitates ribosomes; the precipitate also redissolved on the addition of excess of ribosomes. In both cases precipitation is attributed to electrostatic interaction between oppositely charged macromolecules.

When the polyribosome fraction (2 mg./ml. of buffer) was treated with ribonuclease (60 μ g./ml.) for 15 min. at 20° before zone centrifuging at 0–4° the amount of RNA released from the ribosome was

Fig. 4. Sedimentation patterns of RNA isolated from ribosomes exposed to ribonuclease. The polyribosome fraction (50 mg.) was dissolved in 5 ml. of 0.25 M-sucrose-25 mM-KCl-1 mM-MgCl₂-50 mM-tris-HCl buffer, pH 7.6, and ribonuclease (1.5 μ g.) was added at 22° (final concentration 0.3 μ g. of ribonuclease/10 mg. of polyribosome fraction/ml.). Samples were removed after intervals between 0 and 240 min. and immediately added to 2 vol. of 6 M-guanidinium chloride at 0° to stop the hydrolysis, and RNA was isolated. The sedimentation profile of a dilute solution (about 30 μ g./ml. of 10 mM-potassium phosphate buffer, pH 7.5) was measured before and after exposure to pH 3. The control was prepared by first neutralizing 1 mM-HCl with sufficient potassium phosphate buffer to make the solution 10 mM with respect to phosphate buffer, pH 7.5, and RNA was then added. In this case RNA was always kept at neutral pH values before centrifuging at 42040 rev./min. (Spinco model E ultracentrifuge, analytical D rotor). The position of the sedimenting boundary was measured at 4 min. intervals (—, RNA from untreated ribosomes; Δ , RNA from ribosomes after hydrolysis with ribonuclease for 10 min.; $\circ$, RNA from ribosomes treated with ribonuclease for 240 min.). Another dilution was prepared by first adding RNA to 1 mM-HCl at 0–20° and then, within 2 min., neutralizing with phosphate buffer. The final composition of the solution and the conditions of centrifuging were identical in each, although RNA was briefly exposed to pH 3 to reveal hidden breaks (—, RNA from untreated ribosomes, $\blacktriangle$, RNA from ribosomes treated with ribonuclease for 10 min.; $\bullet$, RNA from ribosomes treated with ribonuclease for 240 min.). The white symbols and black symbols refer to solutions that are identical in composition but differ in the order in which the reagents were added. Values of $S_{20,w}$ were calculated from the movement of the 50%-concentration point and also from the movement of other points on the boundary by the method of Shooter & Butler (1956). For clarity the points (which were calculated at intervals of 0.1 unit of relative extinction) are omitted from the sedimentation profile of RNA isolated from untreated ribosomes before acid treatment.

scarcely altered when the concentration of magnesium chloride in the buffer was increased from 1 mM to 10 mM.

When polyribosomes (2.0 mg./ml.) were treated with ribonuclease (6 μ g./ml.) at 0°, 20° and 30° a fraction of the extinction at 260 nm. was released,

and a well-defined ribosome fraction was found (cf. Fig. 6) in each case. About 20–30% of the extinction was released at 0° and 20° and about 30% at 30° (Fig. 7). The addition of the same amount of ribonuclease to ribosomes at 40–60° led to the immediate formation of a coarse precipitate, which was separated by low-speed centrifuging (3500 rev./min. in the MSE Mistral centrifuge). Fragments of RNA and a small proportion of protein were found in the supernatant. The precipitate was soluble in 4 M-guanidinium chloride and the precipitate obtained at 40° was found to have about 60% of the original extinction at 260 nm. the remaining 40% of the original extinction remained in the supernatant. Zone centrifuging confirmed that the ribonucleoprotein had all aggregated (cf. Fig. 6f). The spectrum of the supernatant fraction was close to that of RNA, and the spectrum of the precipitate in 4 M-guanidinium chloride was consistent with a particle that had lost 40% of RNA and little protein (E_{222}/E_{260} ratio 1.3:1; cf. Fig. 9). Similar results were found at 50° and 60° except that less RNA (50% at 50°; 45% at 60°) was found in the aggregates of nucleoprotein.

Effect of ribonuclease on the sedimentation rate of native subparticles. The ribosome fraction isolated from the cell lysate by differential centrifuging contains a small proportion of so-called 'native' subparticles. When the ribosome fraction (2 mg./ml.) was treated with ribonuclease (10–60 μ g./ml.) both ribosomes and subparticles were found in the same relative proportions on zone centrifuging as in the untreated samples. Thus after extensive ribonuclease treatment native subparticles, like ribosomes, remain as well-defined entities that sediment at about the same rate as the untreated particles. This is in contrast with the effect of ribonuclease on EDTA-subparticles (see Fig. 1). The difference is attributed to tertiary structure. Although EDTA-subparticles are formed without the loss of protein the sedimentation coefficients, $S_{20,w}$, of the smaller and larger subparticles are about 20 s and about 40 s respectively (Arnstein *et al.* 1965) compared with about 40 s and about 60 s for native subparticles. Thus it is inferred that the native subparticles form a much more compact structure in solution than EDTA-subparticles.

Hydrolysis of ribosomes having the RNA moiety in the single-stranded form. The possibility was considered that the RNA moiety may be more readily hydrolysed after conversion into the single-stranded form. Ribosomes (0.34 mg./ml.) in 8% formaldehyde-1 mM-magnesium chloride-10 mM-potassium phosphate buffer, pH 7.5, were heated at 70° for 10 min., cooled and dialysed against sufficient 1 mM-magnesium chloride-10 mM-potassium phosphate buffer, pH 7.5, to decrease the

Fig. 5. Electrophoresis in 10% polyacrylamide gels of RNA from ribosomes treated with ribonuclease. The conditions of hydrolysis and the sedimentation pattern of RNA after hydrolysis are given in Fig. 4. The 10% polyacrylamide gels were prepared by the method of Richards *et al.* (1965) (also see the Experimental section). (a) RNA from the untreated polyribosomes; (b) RNA from polyribosomes treated with ribonuclease for 10 min.; (c) RNA from polyribosomes treated with ribonuclease for 240 min. *A* refers to RNA maintained at about pH 7; *B* refers to samples exposed to pH 3 at 0° for less than 2 min.

formaldehyde concentration to 0.04%. Ribonuclease (3 $\mu\text{g.}/0.34\text{ mg.}$ of ribosomes/ml.; 20 $\mu\text{g.}/\text{mg.}$ of RNA moiety) was then added and the solution was kept at 20° for 1 hr. On filtration through Millipore membranes about 35% of the original extinction at 260 nm. was found in the filtrate, which was found to have an RNA-like spectrum. The sedimentation profile (Fig. 8) of the solution after ribonuclease treatment revealed that the u.v.-absorbing species released did not leave the meniscus and that the ribosome fraction sedimented at about $S_{20,w}$ 70s compared with 80s for the control.

Thermal stability of ribosomes after extensive hydrolysis with ribonuclease. The thermal stability of the untreated polyribosome fraction was checked by measuring the sedimentation profile of samples (1 ml.) of a stock solution (2.5 mg. of polyribosomes/ml. of solvent, namely 0.25 M-sucrose–25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris-hydrochloric acid, pH 7.6) heated at temperatures in the range 0–67.5° for 2–15 min. About half the biological activity was lost when the temperature was increased from 40° to 52.5° and was decreased to 5% after exposure to 67.5°. Nevertheless the sedimentation profile showed that heating led to an increase in the ribosome peak and that the polyribosome peak diminished (cf. Fig. 2). In contrast, when ribosomes were first treated with 10 $\mu\text{g.}$ of ribonuclease/2.5 mg. of polyribosomes/ml.

a coarse precipitate was immediately obtained when the temperature was increased from 20° to 40°. Zone centrifuging showed that about 50% of the total extinction at 260 nm. remained at the meniscus and that there were no other soluble sedimenting species as described above.

The decrease in the thermal stability of ribosomes extensively treated with ribonuclease was found when more dilute solutions were heated. When the ribosome fraction (50 $\mu\text{g.}/\text{ml.}$) is heated in the standard buffer (0.25 M-sucrose–25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris-hydrochloric acid, pH 7.6) the changes in the spectrum closely follow those of the isolated RNA as described in the preceding paper (Cox, 1969) before the protein moiety is precipitated at about 80°. However, precipitation was observed at 45° when the experiment was repeated with ribosomes that had been first treated with ribonuclease (10 $\mu\text{g.}/2.5\text{ mg.}$ of ribosomes/ml.) for 15 min. at 30° or below and then fractionated by zone centrifuging. Aggregation into thread-like particles was noted before precipitation took place.

The precipitate found on heating ribonuclease-treated ribosomes above 40°, which was found to be soluble in 4 M-guanidinium chloride or 1% sodium dodecyl sulphate, had the same properties as the precipitate found on the addition of ribonuclease to ribosomes at elevated temperatures (see above).

Fig. 6. Sedimentation profile of rabbit reticulocyte ribosomes after treatment with ribonuclease. The polyribosome fraction (2.5 mg. of nucleoprotein/ml. of 0.25 M-sucrose-25 mM-KCl-1 mM-MgCl₂-50 mM-tris-HCl buffer, pH 7.6) was divided into six samples (1 ml.). Ribonuclease was added and the samples were kept at 20° for 15 min., layered on to 15-30% (w/v) sucrose gradients and centrifuged for 3.5 hr. at 25000 rev./min. (MSE SW25 rotor). (a) Control (no added ribonuclease); (b) 0.1 µg. of ribonuclease/2.5 mg. of polyribosomes/ml.; (c) 1 µg. of ribonuclease/2.5 mg. of polyribosomes/ml.; (d) 10 µg. of ribonuclease/2.5 mg. of polyribosomes/ml.; (e) 75 µg. of ribonuclease/2.5 mg. of polyribosomes/ml.; (f) 750 µg. of ribonuclease/2.5 mg. of polyribosomes/ml. In (a)-(e) the spectrum of the pellet was the same as that of the ribosome fraction. The pellet obtained in (f) had the spectrum of a nucleoprotein and redissolved on the addition of an excess of the polyribosome fraction. In (b)-(f) the spectrum of fractions 24-29 was similar to that of RNA.

A partial breakdown of the ribosome structure followed by aggregation of the denatured nucleoprotein could explain the results.

Properties of ribosomes that have lost about 25% of

the RNA moiety through enzymic hydrolysis. A concentration of 3 µg. of ribonuclease/mg. of ribosomes appears sufficient to produce the maximum hydrolysis. The properties of RNA-depleted

Fig. 7. Release of RNA from ribosomes on enzymic hydrolysis. The fraction of the original extinction removed from the ribosomes through the action of ribonuclease was obtained from Fig. 6 and similar gradients. $\circ$, Enzymic hydrolysis at 20° for 15 min. before zone centrifuging; $\square$, enzymic hydrolysis in the presence of 5 mM-MgCl₂ at 20° for 15 min. before zone centrifuging; $\blacksquare$, enzymic hydrolysis in the presence of 10 mM-MgCl₂ at 20° for 15 min. before zone centrifuging; $\bullet$, enzymic hydrolysis at 0° for 15 min. before zone centrifuging; $\triangle$, enzymic hydrolysis at 30° for 15 min. before zone centrifuging; ----, % of the original extinction of reticulocyte ribosomal RNA that is soluble in 67% (v/v) ethanol after hydrolysis with ribonuclease for 15 min. at 20° . The solvent was 0.25 M-sucrose–25 mM-KCl–1 mM-MgCl₂–50 mM-tris–HCl buffer, pH 7.6. The solution was made 1% with respect to potassium acetate before the addition of 2 vol. of ethanol.

ribosomes produced by this, or higher, concentrations of enzyme were examined.

RNA/protein ratio. It was found (as described above) that ribosomes that were treated with $3\mu\text{g.}$ or more of ribonuclease/mg. of ribosomes lost about 23% of the RNA moiety as calculated from the distribution of u.v.-absorbing species on zone centrifuging. The changes in the RNA/protein ratio agree with the notion that digestion with ribonuclease leads to the loss of more RNA than of protein. When protein was estimated by the Lowry method with bovine serum albumin as standard the RNA/protein ratio was found to be 46:54 for the control untreated ribosomes compared with 38:62 after enzymic hydrolysis. The buoyant density (measured by the method of Perry & Kelley, 1966) of untreated ribosomes in caesium chloride was found to be 1.60 before and 1.56 after hydrolysis. Both measurements are in accord with the loss of about 20–25% of the RNA from the ribosome with little or no loss of protein.

Ultraviolet-absorption spectrum. Ribosomes were

Fig. 8. Sedimentation profile of rabbit reticulocyte ribosomes heated to 70° in 8% formaldehyde before hydrolysis with ribonuclease. The ribosome fraction (0.34 mg. of ribosomes/ml. of 1 mM-MgCl₂–10 mM-potassium phosphate buffer, pH 7.5) was made 8% with respect to formaldehyde and heated at 70° for 10 min., and then dialysed overnight at 4° against sufficient buffer to decrease the formaldehyde concentration to 0.04%. Ribonuclease ($3\mu\text{g.}/0.34\text{ mg.}$ of ribosome fraction/ml.) was added at 20° . After 1 hr. at 20° a sample was diluted with buffer to $50\mu\text{g.}$ of ribosome fraction/ml. and the sedimentation pattern was obtained on centrifuging at 33450 rev./min. (Spinco model E ultracentrifuge). The movement of the sedimenting boundary was measured at 4 min. intervals.

purified by zone centrifuging as described above and the RNA/protein ratio was measured. The u.v.-absorption spectra of the control and digested fractions were compared (see Fig. 9). As expected the extinction below 250 nm., relative to that at 260 nm., was increased after digestion with ribonuclease. The contribution of protein in the spectrum was calculated: each spectrum was standardized so that E_{250} was 0.9 in each case, i.e. the observed extinction was multiplied by $0.9/E_{250}$; the spectrum of the protein was then obtained by subtracting the spectrum of RNA (see Cox, 1969) from the standardized spectrum of the control and ribonuclease-treated ribosomes. The difference spectra obtained in this way also show that the RNA/protein ratio is decreased after enzymic hydrolysis; the spectrum is consistent with the RNA/protein ratio being about 40:60, in agreement with the chemical composition on the

Fig. 9. Spectrum of ribosomes after treatment with ribonuclease. The solvent was 1 mM-MgCl₂-10 mM-potassium phosphate buffer, pH 7.5. Curve I, untreated ribosomes; curve II, ribosomes treated with ribonuclease (4 μ g./mg. of RNA moiety) and isolated by zone centrifuging; curve III, calculated curve for the extinction of protein (see Fig. 1) of untreated ribosomes; curve IV, calculated extinction of the protein moiety of ribonuclease-treated ribosomes. (It was assumed that the extinction at 250 nm. is the same for both ribosomes and RNA.) Curve I corresponds to that for 100 parts of RNA + 117.5 parts of protein, and curve II corresponds to 100 parts of RNA + 163 parts of protein.

basis of the assumption that the residual RNA retains its partly double-helical structure. The optical rotatory dispersion of the ribonuclease-treated ribosomes (E_{260} 0.7-1.4) in 1 mM-magnesium chloride-10 mM-potassium phosphate buffer, pH 7.5, was measured over the range 220-500 nm. The calculation of the specific rotation was based on the value $E_{1\text{cm}}^{1\%}$ 89 at 260 nm. (cf. $E_{1\text{cm}}^{1\%}$ 112 for intact ribosomes). The observed dispersion curve (Fig. 10) has the form predicted for a particle that has lost about 25% of its RNA, the secondary structure of the remainder being conserved. The differences in the observed and calculated values of $[\alpha]$ below 260 nm. are not considered to be significant. Thus a maximum was found at 276 nm. and a trough at 233-238 nm. (see Fig. 10). Apparently the negative rotation at about 233-238 nm., which is characteristic of native ribosomal protein, was not altered by the loss of RNA.

Electron microscopy (by the late R. C. Valentine).

Fig. 10. Optical rotatory dispersion of ribosomes previously treated with ribonuclease. Ribosomes were treated with ribonuclease (6 μ g./mg. of RNA moiety) and isolated by zone centrifuging. $\circ$, Observed values; ----, values calculated (see Cox, 1969) for a particle of RNA/protein ratio 38:62. The solvent was 1 mM-MgCl₂-10 mM-potassium phosphate buffer, pH 7.5.

The control polyribosome fraction and ribonuclease-treated ribosomes (RNA/protein ratio 38:62) were dissolved (50 μ g./ml.) in 1 mM-magnesium chloride-10 mM-potassium phosphate buffer, pH 7.5, 'fixed' by treatment with 1% formaldehyde at about 20° for 30 min., negatively stained with sodium silicotungstate and then examined in the electron microscope. Electron micrographs (see Plate 2) revealed that, although polyribosomes were absent after ribonuclease treatment, the morphology of the ribosomes was otherwise similar to that of the untreated polyribosome fraction (Plate 1).

X-ray crystallographic analysis (by M. Spencer). X-ray-diffraction patterns of gels of ribonuclease-treated ribosomes showed that the residual RNA has some double-helical character. The diffraction patterns of control and ribonuclease-treated ribosomes were not readily distinguishable.

Properties of RNA isolated from RNA-depleted ribosomes. RNA was isolated from ribosomes that had lost about 25% of the original extinction at 260 nm. as a result of treatment with 3 μ g. (or more) of ribonuclease/mg. of ribosome fraction. To conserve small fragments, the ribosome fraction was made 1% with respect to sodium dodecyl sulphate and deproteinized by treatment (three times) with aqueous phenol, and the phenol was extracted with ether. The nucleotide composition determined (in duplicate) by the method of Katz & Comb (1963). Before hydrolysis the nucleotide composition (expressed as moles/100 moles of nucleotide) was found to be: AMP, 17.5; CMP, 28; GMP, 36; UMP, 18; after hydrolysis the values obtained were: AMP, 17.3 $\pm$ 0.3; CMP, 22.7 $\pm$ 1.6; GMP, 37.7 $\pm$ 0.3; UMP, 21.9 $\pm$ 1.1. The differences in CMP and UMP before and after hydrolysis appear to be significant.

Fig. 11. 'Melting' profile of RNA isolated from RNA-depleted ribosomes. Ribosomes were treated with ribonuclease, and the products were separated as described in Fig. 6. RNA was isolated by the sodium dodecyl sulphate-phenol method and dissolved in 1mM-MgCl₂-10mM-potassium phosphate buffer, pH 7.5. Samples (E_{280} approx. 1.0) were placed in the sample and reference cells. The difference in the spectra of the heated sample and the reference sample kept at 25° was measured. Allowance was made for changes in concentration of RNA due to the thermal expansion of water. A similar result was found when the ribosome fraction from a sucrose gradient was made 1% with respect to sodium dodecyl sulphate. The extinction of the heated sample after cooling was within 3% of the initial value. ○, ΔE_{280} ; ●, ΔE_{260} .

'Melting' profile. RNA isolated from RNA-depleted ribosomes was dissolved in 1mM-magnesium chloride-10mM-potassium phosphate buffer, pH 7.5. One sample was placed in the reference-cell holder of a spectrophotometer and was kept at 25°, and the sample-cell holder contained another sample that was heated at temperatures in the range 25-95°. The difference in the spectra of the heated and reference samples was measured. The increments found in the extinctions at 260 and 280nm. are given in Fig. 11. The increase of about 28% in the extinction at 280nm. from 0.66 to 0.85 at 95° was largely reversed on cooling, when a value of 0.69 was noted, which increased to 0.87 when the temperature was again raised to 95°. A similar 'melting' behaviour was found when a sample of RNA-depleted ribosomes taken from a sucrose gradient was made 1% with respect to sodium dodecyl sulphate and the spectrum was measured at different temperatures. The protein moiety contributes little to the absorption above 240nm. and its contribution to the hypochromism of ribosomes at 260 and 280nm. can be neglected.

Sedimentation profile. RNA from RNA-depleted

ribosomes was found to have $S_{20,w}$ 4s on average after brief exposure to pH 3 (Fig. 12; cf. Fig. 4): this is considered to be a measure of the true molecular size of the fragments. The higher sedimentation coefficients found for samples maintained throughout at about pH 7.5 are attributed to double-helical secondary structure that is sufficiently stable for two fragments to remain united by hydrogen bonds even though a diesterified ribose phosphate bond within the hairpin loop may be hydrolysed. These 'hidden breaks' are revealed once the double-helical secondary structure is destroyed (for discussion see Cox & Kanagalingam, 1967), e.g. by titration with acid to pH 3 (cf. Cox & Littauer, 1963).

Similar sedimentation properties were found when the ribosome fraction taken from a sucrose gradient (e.g. Fig. 6d) was made 1% with respect to sodium dodecyl sulphate. The sedimentation profiles given in Fig. 12 were not altered by increasing the concentration of ribonuclease above 3 μ g./mg. of ribosome fraction/ml., or by altering the temperature in the range 0-30°.

DISCUSSION

Reticulocyte ribosomal RNA is readily hydrolysed by pancreatic ribonuclease at 20°. Extensive degradation is observed with 0.36 μ g. of ribonuclease/mg. of RNA (0.9 μ g./2.5mg. of RNA/ml.; Cox & Kanagalingam, 1967) and hydrolysis is complete when the amount of enzyme is increased tenfold. In contrast, small spherical viruses such as bacterial viruses (Loeb & Zinder, 1961) and animal viruses (Colter, Bird & Brown, 1957; Kaighn, Moscarello & Fuerst, 1964) are not affected by high concentrations of ribonuclease. Ribosomes form an intermediate category in their sensitivity towards ribonuclease.

Low concentrations of ribonuclease (10ng./2.5mg. of polyribosome fraction/ml.) decreased the biological activity of ribosomes (see Table 1), affected their sedimentation profile (see Fig. 2, and also Marbaix & Burny, 1964; Fenwick, 1968) and also produced scissions in the 16s and 30s RNA

EXPLANATION OF PLATES I AND 2

Electron micrographs of rabbit reticulocyte ribosomes and derivatives (by the late R. C. Valentine). Plate 1: untreated polyribosome fraction. Plate 2: polyribosome fraction after hydrolysis with 30 μ g. of ribonuclease/5mg. of RNA for 1 hr. at 20° (this procedure removed about 25% of the RNA moiety from the ribosome); each sample was first fixed with 1% glutaraldehyde for 30 min. at 20° and then negatively stained with sodium silicotungstate before examination by means of a Philips EM200 electron microscope.

R. A. COX

Fig. 12. (a) Sedimentation profile of RNA from RNA-deficient ribosomes. Polyribosomes (2.0 mg./ml. of 0.25 M-sucrose–25 mM-KCl–50 mM-tris-HCl buffer, pH 7.6) were treated with ribonuclease (6 μ g./2 mg. of polyribosome fraction/ml.) for 15 min. at 30°. The products were separated by zone centrifuging as described in Fig. 6. A sample of the ribosome fraction was diluted with 10 mM-phosphate buffer to 25 μ g./ml. and then was made 1% with respect to sodium dodecyl sulphate. One sample was kept at pH 7.6 throughout (curve I; cf. Fig. 4), and another was briefly exposed to pH 3 (cf. Fig. 4) and then neutralized before centrifuging at 59 780 rev./min. (Spinco model E ultracentrifuge, analytical D rotor) (curve II). The progress of the sedimenting boundary was measured at 8 min. intervals (see also Fig. 4). (b) Schematic representation of the conformation of fragments of ribosomal RNA isolated from ribosomes treated with ribonuclease before and after 'hidden' breaks are revealed. (i) Diesterified phosphate linkages within the hairpin loop are hydrolysed but the fragments are held together by residual hydrogen bonds; (ii) double-helical secondary structure is destroyed on titration with acid at 0–25°, so releasing fragments previously held together by hydrogen bonds; (iii) intra-molecular but not intermolecular double-helical secondary structure re-forms when the solution is neutralized (cf. the denaturation of DNA by titration with acid). For discussion see Cox & Kanagalingam (1967).

species (see Figs. 3, 4 and 5). About 20–30% of the total ribosomal RNA can be removed from the ribosome by enzymic hydrolysis (see Fig. 7). The amount of RNA removed is not increased by further addition of ribonuclease up to more than 1 mg. of ribonuclease/mg. of ribosomes, by increasing the temperature from 0° to 30°, or by first converting the RNA moiety into a single-stranded form. The interpretation of the results obtained by treating ribosomes with ribonuclease at temperatures of 40° and above is made difficult because of the aggregation of nucleoprotein that takes place after ribonuclease

treatment. However, even at 60° more than a third of the RNA is found in association with protein. It is suggested that hydrolysis of the RNA moiety decreases the thermal stability of the ribosome.

The sites where RNA is susceptible to attack by ribonuclease are widely distributed throughout the polynucleotide chains because small fragments are produced (see Figs. 4 and 12). The fragments of RNA isolated from RNA-depleted ribosomes were about 35 000 daltons (about 105 residues) on average according to the relation between S and M_w reported by Hall & Doty (1958). Thus it seems that *in situ* RNA from the larger subparticle is susceptible to ribonuclease at about 40 sites/4500 nucleotides, each separated by roughly 400 nucleotides on average. RNA from the smaller subparticle has about 15 sensitive sites/1500 nucleotides.

The high proportion of scissions that are 'hidden' by secondary structure suggests that the sensitive linkages are generally located within hairpin loops. The chance of a scission being 'hidden' increases with size of the loop. On this basis the RNA from the larger subparticle (about 30–35 residues on average/loop; Cox, Gould & Kanagalingam, 1968) might have a higher proportion of hidden breaks than RNA from the smaller subparticle (about 25 residues on average/loop; Cox *et al.* 1968). In accord with this view the smaller subparticle is 'apparently' more susceptible to enzymic hydrolysis than the larger subparticle (e.g. Fig. 3b), whereas, judged on the size of the RNA fragments measured after 'hidden' breaks are revealed (e.g. Fig. 4), the two RNA species appear to be equally susceptible to enzymic hydrolysis. The optical rotatory dispersion of the RNA moiety of the ribosomes, relative to the protein moiety, was decreased after hydrolysis (Fig. 10), showing that the RNA attacked by the enzyme had a well-defined secondary structure. This observation suggests that double-helical segments were hydrolysed.

Although part of the RNA moiety is hydrolysed by ribonuclease the remainder is protected and retains its partly double-helical character, as shown by the X-ray-diffraction pattern and optical properties of ribosomes after ribonuclease treatment and by the 'melting' profile of the isolated RNA. The observation that parts of the 16s and 30s RNA species are protected from ribonuclease is in accord with the observation (Takanami & Zubay, 1964; Takanami, Yan & Jukes, 1965) that part of messenger RNA is protected from ribonuclease and that part of the nascent peptide chain is protected from Pronase (Rich, Eikenberg & Malkin, 1966; Malkin & Rich, 1967). On the basis of hydrodynamic measurements Petermann & Pavlovic (1969) concluded that the larger subparticle of rat liver ribosomes might be a structure with either a deep channel or a hollow centre.

Redman & Sabatini (1966) had earlier suggested that the larger subparticle contains a space or a channel in which the nascent peptide chain grows. The ribosomal RNA that is not susceptible to hydrolysis by ribonuclease might be protected because it forms the lining of a channel or hole within the ribosome. The existence of a channel within the larger subparticle has been proposed on the basis of electron-microscopic studies (Bruskov & Kiselev, 1968; Florendo, 1968). A model of the ribosome in the form of a hollow cylinder with RNA on both the inside and outside surfaces was suggested (Cox, 1967, 1968; Cox & Bonanou, 1969).

The persistence of ribosome-like particles (judged by their sedimentation coefficient and by their appearance as seen under the electron microscope), even after extensive hydrolysis of the RNA moiety (cf. Shakulov, Aitkhodzhin & Spirin, 1962; Santer, 1963; Santer & Smith, 1966), suggests that forces such as protein-protein interactions are important to the stability of the particle. The RNA-depleted ribosomes resemble small spherical viruses in their resistance to further attack by ribonuclease.

The influence of tertiary structure on enzymic hydrolysis is clearly seen from the comparison of the effects of ribonuclease on ribosomes and on EDTA-subparticles (e.g. Figs. 1 and 3). Since EDTA-subparticles retain their full complement of protein subunits (see, e.g., Beeley, Cohen & Keller, 1968), the comparison is valid. The molecular weight of the EDTA-subparticles (Fig. 1), but not of ribosomes (Fig. 6), is affected by small amounts of ribonuclease. It is inferred that there are additional sites exposed on the formation of EDTA-subparticles and that these are the single-stranded regions joining one hairpin loop to another. Chain scission within these single-stranded regions would be expected to lead to an immediate decrease in molecular weight. In the intact ribosome the single strand joining one hairpin loop to another appears to be protected, possibly by the matrix of protein.

A model of the ribosome in which 'the surface of the ribosome consists chiefly of RNA and not protein; and this surface RNA is, by inference, largely double-helical, and the helices probably project outwards from the surface' was proposed by Cotter *et al.* (1967). The present work does not appear to support this model. The high concentrations of ribonuclease used in these studies readily hydrolyse ribosomal RNA with the destruction of double-helical secondary structure and so would be expected to attack exposed double-helical regions of the ribosome. The observation that within the ribosome double-helical secondary structure survives exposure to high concentrations of ribonuclease requires explanation and suggests that a substantial proportion of these regions is inaccessible to the enzyme. This conclusion is

supported by the finding that, even after the RNA moiety is converted into a single-stranded form, its sensitivity towards ribonuclease is not substantially increased. It has also been suggested (Worcel, Goldman & Sachs, 1968) that the RNA moiety forms an inner core of the ribosome. If this is indeed the case the RNA moiety is packed so that a large number of sites are accessible to ribonuclease.

Finally, treatment of ribosomes with ribonuclease suggests that it might be possible to produce specific fragments (cf. Fig. 5) that could be useful in the study of primary sequence and in the study of interaction of ribosomal RNA with ribosomal proteins.

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