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Thermolability of 28S Ribosomal Ribonucleic Acid from the Liver of *Crotalus durissus terrificus* (Ophidia, Reptilia)

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Instability of 28S rRNA of *Crotalus durissus terrificus* liver was observed during hot-phenol extraction: purified 28S rRNA is converted into an 18S RNA component by heat treatment. It was also found that '6S' and '8S' low-molecular-weight RNA species were released during the thermal conversion. This conversion and the release of the low-molecular-weight species were also induced by 8M-urea and 80% (v/v) dimethyl sulphoxide at 0°C. Evidence is presented that this phenomenon is an irreversible process and results from the rupture of hydrogen bonds. The 18S RNA product was shown to be homogeneous by polyacrylamide-gel electrophoresis and by sucrose-density-gradient centrifugation. The base composition of the 18S RNA products obtained by heat, urea or dimethyl sulphoxide treatments was similar. The C+G content of the 18S RNA product was different from that of the native 18S rRNA, but similar to that of 28S rRNA.

The thermolability of the major rRNA species has been mainly described in insect (Applebaum *et al.*, 1966; Greenberg, 1969; Ishikawa & Newburgh, 1972) and mammalian cells (Martin, 1966; Pene *et al.*, 1968; Venkov & Hadjiolov, 1969; Plagemann, 1970). Stevens & Pachler (1972) observed this phenomenon in the unicellular eukaryote *Acanthamoeba castellani*. This thermolability was generally detected as a result of the observation that only the minor rRNA component was recovered when RNA was extracted by the hot-phenol method.

It has been shown that there is a quantitative thermal conversion of the 28S rRNA component into an RNA sedimenting in the 18S region of the gradient. In some organisms this thermal conversion is accompanied by the release of a low-molecular-weight RNA (Pene *et al.*, 1968; Plagemann, 1970). Several attempts have been made to elucidate the mechanism of this phenomenom. Enzyme action has been eliminated by the use of potent inhibitors of nucleases (Applebaum *et al.*, 1966; Pene *et al.*, 1968). The existence of 'hidden breaks' in the native 28S rRNA has been postulated by several authors (Gould, 1967; Spirin & Gavrilova, 1969; Venkov & Hadjiolov, 1969; Koser & Collier, 1971; Stevens & Pachler, 1972). Urea and dimethyl sulphoxide were also able to induce the conversion of 28S rRNA into the 18S RNA product suggesting that the rupture of hydrogen bonds is involved (Applebaum *et al.*, 1966; Plagemann, 1970; Koser & Collier, 1971; Ishikawa & Newburgh, 1972).

However, the conversion of 28S rRNA into an 18S RNA component is not always observed. In HeLa cells (Pene *et al.*, 1968) and in Novikoff hepatoma (Plagemann, 1970) only a slight lowering of the sedimentation coefficient of the major rRNA component was found.

The unique information on this subject in Reptilia is the observation that only the 18S rRNA is recovered when the RNA of *Crotalus durissus terrificus* is extracted by the hot-phenol method (De Lucca, 1970). The present paper is an effort to characterize this phenomenon in the liver of the South-American rattlesnake.

Materials and Methods

Materials

Chemicals. All chemicals were reagent grade where possible. Phenol was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. and distilled immediately before use. Sodium dodecyl sulphate was from BDH Chemicals Ltd., Poole, Dorset, U.K. Bentonite was from Sigma Chemical Co., St. Louis, Mo., U.S.A. and was purified as described by Fraenkel-Conrat *et al.* (1961). For polyacrylamide-gel electrophoresis, acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were obtained from Canalco, Bethesda, Md., U.S.A. Dimethyl sulphoxide and urea were from J. T. Baker Chemical Co., Phillipsburg, N.Y., U.S.A. Deoxyribonuclease I (bovine pancreas, ribonuclease-free)

was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Snakes. South-American rattlesnakes (*Crotalus durissus terrificus*) of both sexes (300–400g body wt.) were used in all experiments.

Methods

Preparation of total RNA. The animals were killed by decapitation, the liver was quickly removed and the RNA extracted by the cold- and hot-phenol methods. The cold-phenol method was performed as described previously (De Lucca & Imaizumi, 1972). The RNA obtained by this procedure was suspended (approx. 2mg/ml) in 0.01M-Tris–HCl buffer, pH7.4, containing 0.05M-NaCl and 2mM-$MgCl_2$. Stock solution of deoxyribonuclease was added to a final concentration of 5μg/ml and the mixture was left for 20min at 4°C. After this period potassium acetate and ethanol were added to final concentrations of 2M and 25% (v/v) respectively. After being left for 6h at −15°C the precipitated RNA was collected by centrifugation and resuspended in 1mM-$MgCl_2$ in distilled water. The precipitation was repeated twice.

The hot-phenol method was performed as described by Scherrer & Darnell (1962) except that bentonite was used instead of polyvinyl sulphate as an inhibitor of ribonuclease. In this procedure the treatment with deoxyribonuclease was omitted because the test of Burton (1956) indicated a negligible contamination by DNA.

In both methods of extraction the yield was about 3mg/g of fresh tissue. The concentration of RNA was measured as the E_{260} in a Zeiss model PMQII spectrophotometer, and 1 E_{260} unit was considered to be 40μg of RNA/ml. Only the preparations of RNA with an E_{260}/E_{280} ratio of about 1.9–2.0 were used.

Isolation of 28S and 18S rRNA. Samples of total RNA (approx. 1mg) were layered on linear 5–20% or 15–30% (w/v) sucrose gradients; the sucrose solutions were made in 0.02M-Tris–HCl buffer, pH7.6, containing 0.1M-NaCl and 1mM-EDTA. The gradients were centrifuged in a SW-25.1 rotor in a Spinco model L ultracentrifuge at 25000rev./min at 4°C for 16h. Fractions (1ml) were collected dropwise and their E_{260} values were measured. The three fractions with the highest E_{260} values in each of the 28S and 18S peaks were precipitated, after NaCl had been increased to a final concentration of 0.1M, with 3vol. of ethanol at −15°C for at least 12h. The RNA was collected by centrifugation and stored at −15°C under 80% (v/v) ethanol until further use.

Heat treatment. Samples of RNA solution were heated in 0.01M-sodium acetate buffer, pH5.0, or 0.01M-Tris–HCl buffer, pH7.6, containing 0.01M-NaCl. The heating was done in stoppered test tubes at 60°C for 3min and after this treatment the samples were rapidly cooled in an ice bath. The heated RNA was analysed by sucrose-density-gradient centrifugation as described previously (De Lucca & Imaizumi, 1972) and by polyacrylamide-gel electrophoresis (Loening, 1967). Gels were fixed in acetic acid and scanned at 260nm in a Beckman model Acta III spectrophotometer.

Treatment with urea or dimethyl sulphoxide. In both cases the RNA was dissolved in 0.01M-sodium acetate buffer, pH5.0, containing 0.05M-NaCl. Stock solutions of urea and dimethyl sulphoxide were added to final concentrations of 8M and 80% (v/v) respectively. After an appropriate time of incubation at 0°C the RNA was precipitated with 4vol. of ethanol at −15°C for at least 12h, collected by centrifugation and washed once with 80% (v/v) ethanol. The samples of RNA submitted to these conditions were analysed as described under 'Heat treatment'.

Base composition. This analysis was done by the method of Katz & Comb (1963).

Results

Effect of the hot-phenol extraction on 28S rRNA

Fig. 1 shows the sedimentation profiles of total RNA extracted at 4° and 60°C. When RNA was extracted by the hot-phenol method only 18S rRNA was recovered. Electrophoresis on 7.5% (w/v) polyacrylamide gel of the 4° and 60°C preparations indicates that RNA obtained by the hot-phenol method has in addition to the 4S and 5S RNA species, a third type of low-molecular-weight RNA (Fig. 2). This type of RNA will be referred to as '6S' which has also been described in other eukaryotic cells (Venkov & Hadjiolov, 1969; Pene *et al.*, 1968; Sy & McCarty, 1970; Plagemann, 1970).

Heat treatment of total RNA extracted at 4°C

After heating, the total RNA was analysed by electrophoresis on 2.5% (w/v) polyacrylamide gel (Fig. 3). This result indicates a decrease in 28S rRNA and a corresponding increase in the 18S RNA component. Another type of low-molecular-weight RNA which has a lower electrophoretic mobility than that of '6S' RNA was also observed. This type of RNA is referred to below as '8S' RNA.

Heat treatment of purified 28S and 18S rRNA

To study further the effect of temperature, experiments were made with purified 28S and 18S rRNA. The findings (Fig. 4) indicate that heating of 28S rRNA at 60°C for 3min converts it into a fraction sedimenting in the 18S region of the sucrose gradient.

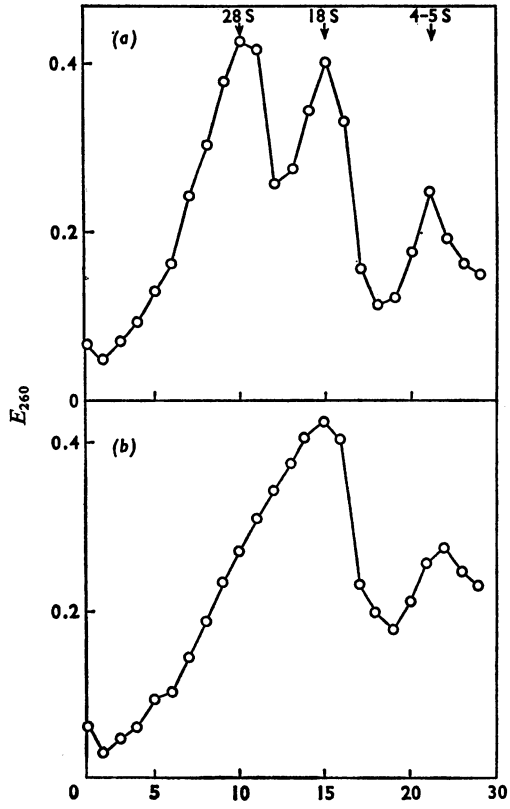

Fig. 1. *Sucrose-density-gradient sedimentation profiles of RNA extracted at (a) 4°C and (b) 60°C*

Total RNA was extracted by the cold- and hot-phenol methods as described in the Materials and Methods section and layered on linear 5–20% (w/v) sucrose gradients. The centrifugation was for 4h at 39000rev./min in the SW-39 rotor of a Spinco model L ultracentrifuge.

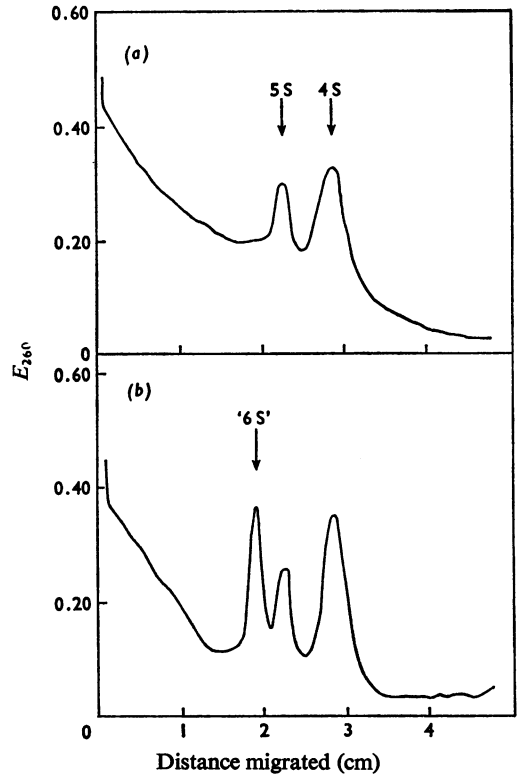

Fig. 2. *Polyacrylamide-gel electrophoresis of RNA extracted at (a) 4°C and (b) 60°C*

Total RNA was dissolved at a concentration of approx. 1mg/ml in Loening's (1967) electrophoresis buffer and samples of 50μl were applied to 7.5% (w/v) polyacrylamide gels. Electrophoresis was for 1h at 5mA/gel. Other details were as described in the Materials and Methods section.

The 18S rRNA, however, was stable under these conditions. The thermal conversion of 28S rRNA was also observed in the presence of inhibitors of nucleases such as sodium dodecyl sulphate, bentonite and macaloid, all at a concentration of 0.1%.

It was also found that heat treatment of purified 28S rRNA releases the '6S' and '8S' RNA species.

In some experiments, the 28S rRNA was heated in 0.15M-NaCl–0.015M-sodium citrate and the sample was gradually cooled at room temperature. Sedimentation analysis on a sucrose gradient showed that there was no back-conversion of the 18S RNA product into 28S rRNA.

Effect of salt concentration and pH on the thermal conversion of 28S rRNA

These experiments were performed with total RNA extracted at 4°C. Fig. 5 shows that heat treatment was more effective when the buffer contained a low salt concentration. Essentially no differences were found in the experiments done at pH5.0 and at pH7.6.

Conversion induced by urea and by dimethyl sulphoxide

To minimize the action of residual nucleases, all these experiments were done at 0°C. Fig. 6 indicates that 8M-urea induces a complete conversion in 2h, whereas 80% (v/v) dimethyl sulphoxide does this in 1min. The product of the conversion induced by these

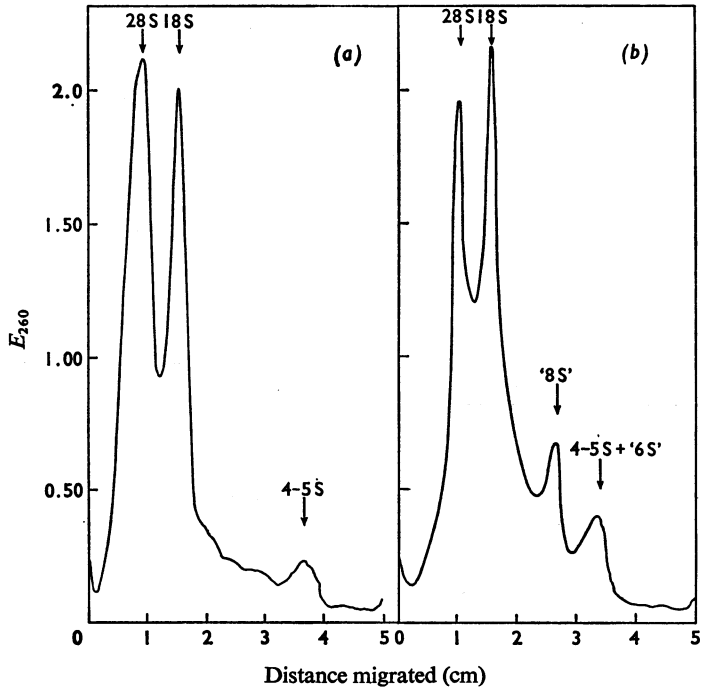

Fig. 3. *Effect of heating on total RNA extracted at* 4°C

The extraction and heat treatment of RNA were as described in the Materials and Methods section. Samples of 50μg of unheated RNA (*a*) and heated RNA (*b*) were applied to 2.5% (w/v) polyacrylamide gels. Electrophoresis was for 1h at 4mA/gel.

Table 1. *Base composition of* 28*S rRNA*, 18*S rRNA and* 18*S RNA product*

Results are expressed as means±S.E.M. of four determinations. Details of RNA hydrolysis and base analysis are described in the Materials and Methods section.

Type of RNA	Content (mol/100mol) U	A	C	G	C+G
28S rRNA	20.1±0.6	15.0±0.6	27.2±1.0	37.7±1.0	64.9
18S rRNA	22.2±0.9	19.6±1.4	25.9±1.2	32.2±0.9	58.1
18S RNA product after:					
Heat treatment	20.6±1.8	14.7±1.5	25.2±1.2	39.5±0.3	64.7
Urea	19.5±0.7	17.3±0.6	25.2±2.1	38.0±1.9	63.2
Dimethyl sulphoxide	18.7±0.5	16.6±1.2	26.3±0.8	38.5±2.0	64.8

treatments appeared in the 18S region of the sucrose gradient.

Electrophoresis on 7.5% (w/v) polyacrylamide gel of 28S rRNA treated with 8M urea or 80% (v/v) dimethyl sulphoxide indicates that both treatments also release the '6S' and the '8S' RNA.

Characterization of the 18*S product*

It was decided to compare the base composition of the native 18S rRNA and the 18S RNA product obtained by heating and by treatment with urea or dimethyl sulphoxide. These treatments were used to obtain a complete conversion of purified 28S rRNA.

The results (Table 1) indicate that the 18S RNA product obtained by all three treatments has a similar C+G content. It should also be noted that the C+G content of the 18S RNA product is different from that of the native 18S rRNA, but similar to that of the 28S rRNA.

Discussion

The lability of 28S rRNA from the liver of *Crotalus durissus terrificus* was shown by brief treatment at

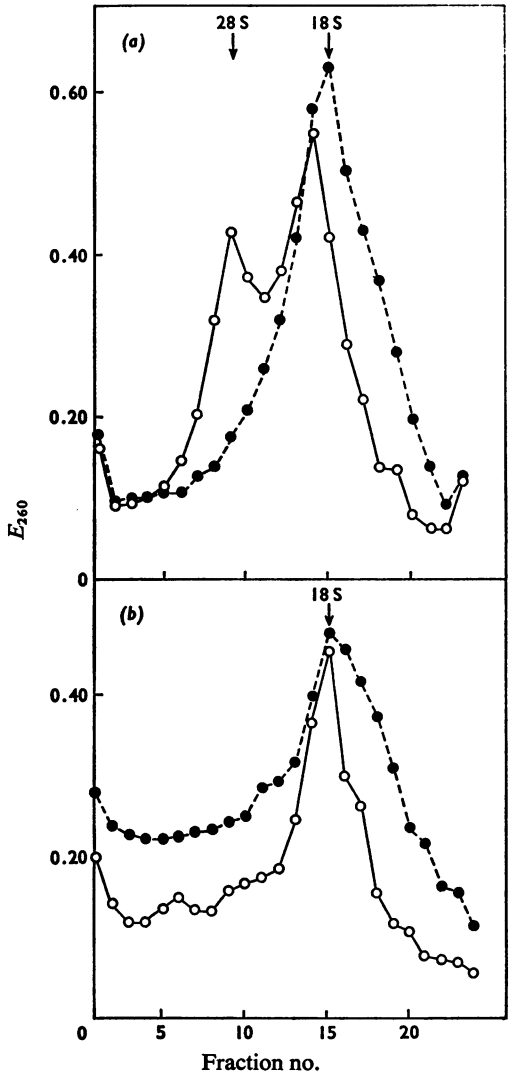

Fig. 4. *Effect of heat on purified 28S and 18S rRNA*

(*a*) Mixture of purified 28S (27μg) and 18S (40μg) rRNA before and after heating; (*b*) purified 18S rRNA (40μg) before and after heating. The rRNA species were extracted, purified and heated as described in the Materials and Methods section. The centrifugation on 5–20% (w/v) linear sucrose density gradients was done as described in Fig. 1. ○, Unheated RNA; ●, heated RNA.

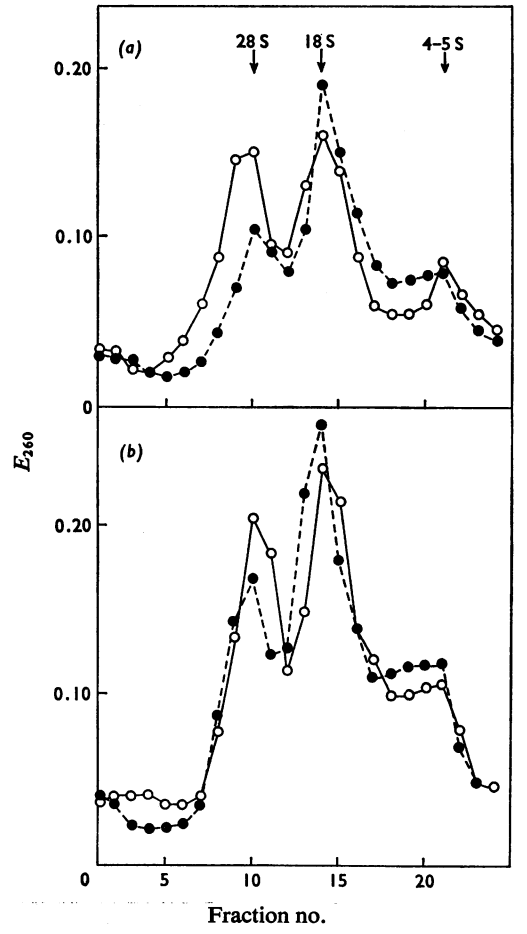

Fig. 5. *Effect of salt concentration and pH on the thermal conversion of 28S rRNA*

Total RNA was extracted by the cold-phenol method as described in the text. After precipitation of the RNA, samples were dissolved in 0.01M-sodium acetate buffer, pH5.0, containing 0.01M- or 0.20M-NaCl (*a*) and in 0.01M-Tris–HCl buffer, pH7.6, containing 0.01M- or 0.20M-NaCl (*b*). After heating, the samples were centrifuged on 15–30% (w/v) linear sucrose density gradients for 5h at 39000rev./min in the SW-39 rotor of a Spinco model L ultracentrifuge. ○, With 0.20M-NaCl; ●, 0.01M-NaCl.

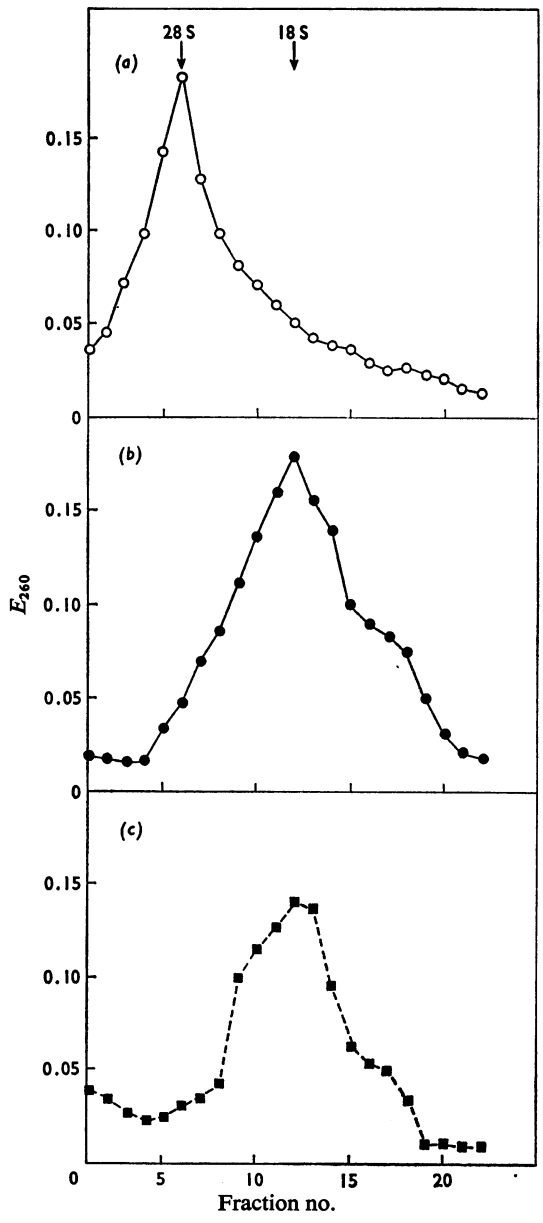

Fig. 6. *Effect of urea or dimethyl sulphoxide on purified 28S rRNA*

The 28S rRNA was purified and treated as described in the Materials and Methods section. The centrifugation was performed as described in Fig. 1. ○, Control; ●, 8M-urea; ■, 80% (v/v) dimethyl sulphoxide.

60°C or with 8M-urea or 80% (v/v) dimethyl sulphoxide. All these treatments converted 28S rRNA into an 18S RNA component and released the '6S' and '8S' low-molecular-weight RNA species. The main characteristics of the lability of 28S rRNA of this reptile were similar to those described for other classes of animals.

The findings reported here suggest that the 28S rRNA from rattlesnake liver is not composed of two similar sections as reported for the large rRNA of *Escherichia coli* (Fellner & Sanger, 1968), yeast (Van den Bos & Planta, 1970) and *Tetrahymena pyriformis* (Bostock *et al.*, 1971).

The hypothesis that this phenomenon is due to enzymic action was eliminated by heating purified 28S rRNA in the presence of strong inhibitors of nucleases. Moreover the conditions of extraction were also inhibitory for nuclease activity.

The following results suggest that the rupture of hydrogen bonds is involved in this phenomenon: (*a*) conversion induced by urea or dimethyl sulphoxide; (*b*) the degree of the thermal conversion was dependent on the salt concentration.

It should be emphasized that the conditions used in these experiments are not sufficient to rupture the phosphodiester bond (Koser & Collier, 1971; Ishikawa & Newburgh, 1972). It seems more likely that 28S rRNA of rattlesnake liver contains 'hidden breaks' in the polymer chain which are revealed by alteration of its secondary structure. There are evidences that these breaks appear *in vivo* during processing of the rRNA precursor molecule (Applebaum *et al.*, 1966; Pene *et al.*, 1968; Sy & McCarty, 1971; Ishikawa & Newburgh, 1972).

The homogeneity of the 18S RNA product was examined by electrophoresis on polyacrylamide gels and centrifugation on sucrose gradients. The 18S RNA product was homogeneous at least under the conditions used in those experiments. However, the sedimentation profiles (Figs. 4 and 6) indicate that the 18S RNA peak is broad and this fact is probably due to the presence of '8S' low-molecular-weight RNA.

The base-composition analysis indicates that the 18S RNA product is distinct from the native 18S rRNA. As pointed out for the silkmoth *Hyalophora cecropia* (Applebaum *et al.*, 1966), the fact that the product and the native rRNA have the same sedimentation coefficient is fortuitous.

The 18S RNA products obtained by heat, urea or dimethyl sulphoxide treatments had the same behaviour on sedimentation analysis and similar C+G content. These findings strongly suggest that the polynucleotide chain is always cleaved at the same point or in the region nearby.

It is also noteworthy that the thermal conversion of 28S rRNA from rattlesnake liver seems to be an irreversible process, as observed in the wax moth *Galleria mellonella* (Ishikawa & Newburgh, 1972).

The significance of the low-molecular-weight RNA associated with 28S rRNA of *Crotalus*, presumably by hydrogen bonds, is still unclear. It has been sug-

gested that this type of low-molecular-weight RNA may have a role in the three-dimensional configuration of the large rRNA (Pene *et al.*, 1968; Sy & McCarty, 1971) and have some function in protein or ribosome biosynthesis (King & Gould, 1970).

It should be emphasized that the conversion is always accompanied by the release of '6S' RNA. Probably, this phenomenon is universal and this low-molecular-weight RNA was not observed in some organisms because of problems of methodology employed.

Finally, the discontinuity of the large rRNA has been described in several classes of animals and now in the Reptilia. Since this phenomenon seems to be widespread, it may be of structural importance in the ribosome, as suggested by Stevens & Pachler (1972).

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