HIGHLY POLYMERIZED RIBONUCLEIC ACID: PREPARATION FROM LIVER AND DEPOLYMERIZATION

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The degree of polymerization of native ribonucleic acid (RNA) has received relatively little attention. No highly polymerized RNA from mammalian tissue has been reported, probably because degradation had occurred under the drastic procedures generally used in the isolation of RNA. The freshly isolated RNA of tobacco mosaic virus, prepared under mild procedures by Cohen and Stanley (1), had a molecular weight of approximately 300,000; when alkali was used in the extraction, it had a molecular weight of 15,000, close to that of commercial yeast nucleic acid. Calf spleen RNA, isolated by Volkin and Carter (2), had a sedimentation constant indicative of a molecular weight of 30,000 to 50,000.

In the present investigation, cytoplasmic RNA was isolated from calf and rat liver. The nucleic acid had the physical properties of a high polymer with a molecular weight in the order of 300,000, approximating that of nucleic acid from tobacco mosaic virus. The RNA was depolymerized by x-radiation, chemically generated free radicals, and sonic vibration. The results suggest that native cytoplasmic RNA exists in a more highly polymerized state than had previously been supposed.

EXPERIMENTAL

Preparation of Cytoplasmic Extract—Calf liver was used for Preparation 1; livers from normal male albino rats varying from young to full grown animals were used for the others. Freshly excised livers in 100 gm. lots were frozen in dry ice-acetone for 10 to 20 minutes and passed, while in a semifrozen state, through a meat grinder into 2.5 volumes of 0.4 M NaCl, as advocated by Luck (3). All work was performed in the cold as far as possible to minimize ribonuclease activity. The ground liver suspension was homogenized in a Waring blendor for 10 seconds and stirred at relatively low speed for 30 minutes. The homogenate in 100 ml. bottles was centrifuged at 3000 r.p.m. in an International, type SB, size 1 model, equipped with an angle head. The supernatant was recentrifuged 25 minutes to insure removal of nuclei and cellular débris. The sediment was extracted with 2.5 volumes of 0.14 M NaCl for 15 minutes and centrifuged; the supernatant was recentrifuged and combined with the main
cytoplasmic extract. The volume of the extract from 100 gm. of liver was about 325 ml.

Isolation of RNA from Cytoplasmic Extract—A modified method of Volkin and Carter (2) was used. The extract was adjusted to 4 M concentration with respect to guanidine hydrochloride (Eastman Kodak, practical grade), and the pH was brought to 6.5 to 7.0 by dropwise addition of dilute NaOH with stirring. The extract was maintained at a temperature of 38–40° for 1 hour, during which time the greater part of the protein became solubilized. The solution was chilled rapidly to about 2° and placed in the refrigerator for 1 hour. The amorphous gelatinous precipitate that formed was centrifuged and washed once with 4 M guanidine hydrochloride and twice with 0.14 M NaCl.

The washed nucleic acid-protein precipitate (which, in contrast to that from calf spleen reported by Volkin and Carter, could not be dissociated by exhaustive dialysis against water in previous experiments) was taken up in 35 ml. of 0.14 M NaCl, and the suspension in a Pyrex test-tube was placed in a boiling water bath, according to the method of Cohen and Stanley (1), and stirred. Within 1 minute the suspension reached 90° and was maintained between 90° and 100° for 2 minutes, during which time flocculation of the precipitate occurred. (In order to ascertain whether the brief period of heating was producing an artifact by causing polymerization of the RNA, several preparations were made without heating; although a high polymer was obtained, the N:P ratios, in general, were not as low as those in preparations in which the protein had been denatured by heating.) The suspension was chilled to about 2° and the precipitate then appeared somewhat gelatinous and the suspension quite viscous. Deproteinization was carried out according to the method of Sevag et al. (4). An equal volume of chloroform-butanol (4:1) was added to the suspension and the mixture was shaken for 6 hours until partial dissociation occurred. The emulsion was centrifuged, and three layers were observed above the chloroform phase: an opalescent solution of RNA contaminated with protein, a bulky layer of undisassociated nucleic acid-protein complex, and a thin, tan, interfacial layer of protein. The two top layers were removed and combined; this suspension was shaken with chloroform-butanol for 2 hour periods three or four times until there was no interfacial "skin," which sometimes consisted of fibers up to 1 cm. in length, after centrifugation. 2 volumes of 95 per cent ethanol were added to the aqueous phase, and the bulky gelatinous precipitate of RNA was centrifuged. The RNA was taken up in water and reprecipitated from alcohol to obtain a salt-free product. After centrifugation, the RNA precipitate was taken up in 35 ml. of water to make an approximately 1 per cent concentration. The noticeably viscous solution was centrifuged at high speed to remove a
small amount of insoluble material. The yields approximated 400 mg. of RNA from 100 gm. of liver.

Physicochemical Properties—The lyophilized RNA, a lustrous, white, non-hygroscopic solid, readily dissolved in water to form a clear viscous solution. The RNA preparations, except Preparation 6B, showed higher N:P ratios (Table I) than those of yeast RNA, which were in agreement with the values found by Brues, Tracy, and Cohn (5) for normal rat liver. Preparation 1 from calf liver had an N:P ratio essentially the same as that found by Chargaff et al. (6) for calf liver RNA. All preparations showed an intense blue ring indicative of much pentose when tested with the β-naphthol reagent (7); the diphenylamine reaction for deoxyribonucleic acid (DNA) was negative; the biuret reaction for protein was negative except in cases of a high N:P ratio. Ultraviolet absorption spectra were typical of nucleic acids with maximum absorption at 2600 Å. Some of the RNA preparations exhibited about one-tenth the intensity of streaming birefringence of highly polymerized DNA solutions examined with the same apparatus. RNA, precipitated from solution by 3 per cent trichloroacetic acid and centrifuged, formed a white plastic pellet which was drawn into a thread that possessed considerable elasticity. These threads, when allowed to air dry under tension, lost their elasticity and became brittle upon further drying. The RNA threads exhibited orientation under the polarizing microscope.

Anomalous viscosity, which varied considerably with the individual preparation and concentration, was shown by solutions of liver RNA (Fig. 1). Relative viscosities were measured in a Bingham-Jackson (8) type of viscometer at an external pressure ranging from 5 to 60 mm. of mercury and
were plotted against the mean velocity gradient in the manner of Edsall and Mehl (9). The viscosities decreased with time, indicating some degradation or change in molecular configuration in the freshly prepared RNA solutions. The viscosities decreased markedly after adding NaCl, or after heating. After addition of NaCl to a 0.1 M concentration, the relative viscosity of a 0.50 per cent solution of Preparation 5 decreased about 50 per cent and the anomalous viscosity disappeared completely; the viscosity then remained constant over a 5 day period. The relative viscosity of a 1.33 per cent solution of one preparation subjected to a temperature of 95° for 2 minutes decreased 30 per cent, and the anomalous viscosity was almost completely lost.

Sedimentation velocities were measured by means of a Beams type...
air-driven ultracentrifuge, generally at speeds of 42,000 to 52,000 r.p.m. Measurements were made in unbuffered NaCl solutions at pH 5 to 6 and in a few instances in water. The RNA was decidedly polydisperse in a saline medium; in water, however, it was essentially monodisperse throughout the run, which indicated a considerable amount of molecular aggregation. (Aggregation in water solution and dispersion in the presence of salt were also indicated by the viscosity behavior described above for RNA.) Sedimentation-velocity constants (Table I) were calculated from the movement of the peak of the curves. Sedimentation data at three concentrations of rat liver RNA, Preparation 2, are given in Table II.

The average molecular weight of calf liver RNA (Preparation 1) was estimated from the sedimentation constant in 0.07 M NaCl at a concentration of 0.46 per cent; a value of 0.48, found by Bacher and Allen (10) for yeast sodium ribonucleate, was used as the apparent specific volume.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Solvent, NaCl</th>
<th>$s 	imes 10^{-12}$</th>
<th>$s_{10,0}$</th>
<th>$s_{20}$</th>
<th>Viscosity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.07</td>
<td>9.8</td>
<td>8.7</td>
<td>24.3</td>
<td>2.78</td>
</tr>
<tr>
<td>0.50</td>
<td>0.07</td>
<td>11.6</td>
<td>10.2</td>
<td>16.2</td>
<td>1.58</td>
</tr>
<tr>
<td>0.25</td>
<td>0.07</td>
<td>11.5</td>
<td>10.3</td>
<td>12.7</td>
<td>1.24</td>
</tr>
<tr>
<td>0.00</td>
<td>Extrapolated</td>
<td>10.4</td>
<td>10.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The molecular weight was calculated to be 264,000, corrected for viscosity of the solvent, and 580,000, corrected for viscosity of the solution according to Lauffer (11). The axial ratio was found to be 70:1; the intrinsic viscosity was 319.

Depolymerization

$X$-irradiation—RNA solutions were irradiated by means of a General Electric Maximar x-ray therapy machine (self-rectifying) operating at 250 kv. (peak) and 15 ma., rate 576 r. per minute, total dose 10,000 r. In addition to the inherent filtration of 3 mm. of aluminum, a duralumin filter of 0.001 inch thickness covered the small glass dish which held the samples. The distance from the sample to the focal point was 23.8 cm. A marked drop in viscosity occurred within 30 minutes after irradiation of a 0.40 per cent solution of calf liver RNA, Preparation 1 (Fig. 2); the viscosity continued to fall over a 24 hour period, which suggests that depolymerization occurred through a free radical type of mechanism. No significant amounts of dialyzable components were produced as a result of this irradiation. Rat liver RNA was depolymerized similarly to calf liver RNA.
Free Radicals—A loss of viscosity of liver RNA solutions occurred after treatment with chemically generated free radicals produced by the reduction of $10^{-3}$ m H$_2$O$_2$ by $10^{-3}$ m FeSO$_4$. The viscosity of a 0.50 per cent solution of Preparation 7 suffered a 50 per cent reduction in viscosity within 30 minutes after addition of the reagents, despite the high N:P ratio (2.44) and the possibility of protection by contaminating protein. A considerable loss of viscosity of RNA solutions was caused by FeSO$_4$ alone in concentrations as low as $10^{-4}$ m. The viscosity of RNA was affected only slightly by $10^{-3}$ m H$_2$O$_2$, as had been found by Limperos and Mosher (12) for DNA solutions.

Sonic Vibration—7 to 8 ml. samples of a 0.25 per cent RNA solution, Preparation 5, were subjected to sonic vibration for different periods of time at a temperature range of 14.7–16.0° in a sonic oscillator, type R-22-3, Raytheon Manufacturing Company, Waltham, Massachusetts. The frequency was 9000 cycles per second; intensity data are not available in absolute units, but the maximum output of the oscillator was used. A marked decrease in viscosity occurred after 20 and 40 minutes of sonic vibration. Vibration for 60 minutes resulted in a viscosity drop essentially the same as that obtained after 40 minutes.

**DISCUSSION**

Complete dissociation of the RNA-protein complex in this investigation was difficult. Unlike nucleoprotein from tobacco mosaic virus, the complex did not undergo spontaneous dissociation after heat denaturation, which suggests that cytoplasmic RNA is attached to its protein by a stronger bond than RNA from tobacco mosaic virus.

Short exposure of nucleoprotein to high temperatures may effect not
gross denaturation, but only an unfolding of the molecule to a spatial configuration compatible with further surface denaturation and dissociation, such as results upon shaking with chloroform. A change in the shape of the protein component of a nucleoprotein may be reflected in a similar change in the closely bound nucleic acid; therefore, the method and extent of denaturation of the protein are probably a major factor in determining the size and shape of the dissociated nucleic acid molecule.

The fact that liver cytoplasmic RNA was found to be heterogenous in the ultracentrifuge does not necessarily indicate that degradation occurred during the relatively mild isolation procedure. It may indicate that the physiologically dynamic RNA is naturally heterogenous.

The depolymerization of RNA in vitro in this investigation suggests that RNA, like DNA (13), is also depolymerized by radiation in vivo. If this is so, the depolymerization of cytoplasmic RNA in vivo to low molecular weight, diffusible nucleotides may offer a partial explanation of the metabolic disturbances in cells after irradiation. In support of this statement it should be noted that a marked increase of cytoplasmic pentose nucleotides, after exposure of cells to ionizing radiation, was found by Mitchell (14), and the combined effects of pentose nucleotides injected into mice were reported by Parsons, Gulland, and Barker (15) "to reproduce with singular exactness the systemic effects induced in mice by x-radiation." Low molecular weight RNA and ribose mononucleotides were found by Zittle (16) to inhibit certain oxidation-reduction enzyme systems; this suggests one method by which radiation may cause metabolic disturbances.

SUMMARY

Highly polymerized ribonucleic acid has been prepared from calf and rat liver. The procedure involves the maintenance of the pH between 6 and 7, the precipitation of a nucleic acid-protein complex from the cytoplasmic extract according to the method of Volkin and Carter, heat denaturation of the protein for 2 minutes at 100°, and removal of the protein by shaking with chloroform.

The average apparent molecular weight of the RNA of a typical preparation, calculated from the sedimentation-velocity constant, was 264,000, corrected for viscosity of the solvent, and 580,000, corrected for viscosity of the solution. The axial ratio was 70:1, and the intrinsic viscosity was 319. Anomalous viscosity and moderate streaming birefringence were exhibited by the RNA solutions.

The RNA was depolymerized by x radiation, chemically generated free radicals, and sonic vibration.
BIBLIOGRAPHY

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