RIBONUCLEIC ACID FROM *ESCHERICHIA COLI*

PREPARATION, CHARACTERIZATION AND PHYSICAL PROPERTIES

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(Received July 19th, 1958)

SUMMARY

A method has been developed for the isolation of RNA from *E. coli* "protoplasts", by extraction with a phenol–water mixture. The RNA preparation contained no detectable DNA and only traces of proteins and polysaccharides. Sedimentation in the ultracentrifuge yielded three boundaries. The two faster moving components were separated from the slower one by $(NH_4)_2SO_4$ precipitation from a phenol-saturated water solution. From sedimentation, viscosity and light-scattering data the molecular weight was estimated to be of the order of one million. The RNA preparations in their viscosity behavior closely resemble coiling synthetic polyelectrolytes, and behave quite unlike DNA. The viscosity behavior, birefringence of flow, and potentiometric titration data are discussed in terms of a single contractile coil model.

The $\varepsilon(P)_{260\,m\mu}$ values of the isolated material in 1 *M* phosphate (pH 7.1) were about 7400. Alkaline hydrolysis or polyribonucleotide phosphorylase action brought about an increase in absorption ranging from 56 to 59 % while pancreatic ribonuclease caused a 28–31 % increment. At very low concentrations of RNAase the change in optical density remained constant, while viscosity decreased rapidly with time.

INTRODUCTION

The purpose of this work was the isolation of a high molecular weight RNA from *E. coli* and the study of its physical properties. The extraction of RNA from bacteria has been hampered because of the difficulty in: (a) breaking the cell wall, which prevented the use of mild extraction procedures; and (b) rapid inactivation of the cell ribonuclease. Thus JONES *et al.*[1] used 2 % sodium cholate in saline at 60° to extract the nucleic acids of *Aerobacter aerogenes*. Though the bacterial RNA-hydrolyzing enzymes were inactivated, the high temperatures employed may have caused some damage to the RNA obtained. The use of phenol for the isolation of bacterial RNA was first indicated by the work of WESTPHAL *et al.*[2]. Water-saturated phenol was also used by

The abbreviations used are: adenosine diphosphate, ADP; deoxyribonucleic acid, DNA; ethylenediaminetetraacetic acid, Versene; ^{32}P-labeled inorganic ortho phosphate, ^{32}P; perchloric acid, PCA; ribonuclease, RNAase; ribonucleic acid, RNA; tris(hydroxymethyl)aminomethane, Tris; tobacco mosaic virus, TMV.

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GIERER AND SCHRAMM[3] for the successful isolation of infectious RNA from TMV. Their work indicated that phenol could be used for the isolation of a biologically-active RNA. KIRBY[4] also used a phenol and water mixture for the isolation of mammalian RNA in good yield. In addition he found that pancreatic RNAase was completely inhibited by treatment with phenol at 20°.

We have confirmed the results of WESTPHAL *et al.*[2] for the isolation of RNA from *E. coli* cells by treatment with a phenol–water mixture. Some RNA was extracted in the water phase, but the yield was very small and the preparations heavily contaminated with polysaccharides. We have shown[5] that better yields of RNA can be obtained if the cell wall is removed prior to the phenol treatment. A very mild method for the degradation of bacterial cell walls involves the use of the enzyme lysozyme. This method was successfully used for the isolation of DNA by VENDRELY *et al.*[6] and for biologically active RNA by HUNTER AND BUTLER[7]. We also have used lysozyme to degrade the cell wall prior to RNA extraction. To avoid RNA degradation by RNAase during lysozyme action the digestion was carried out at 0° and in the presence of 1 *M* sucrose; thus the cells did not lyse and protoplasts were obtained. Treatment of the protoplast suspension with phenol gave high yields of RNA with very small amounts of impurities. Moreover, the molecular weight of the RNA was found to be of the order of 10^6, which is the same order reported for viral[8,9,10] and ascites-tumor RNA[11] (in three of these preparations[9,10,11] phenol extraction was used). These values are higher than those found for yeast[12] and mammalian tissue[13–15] RNA preparations.

The physical properties of the highly polymerized RNA obtained are reported in detail in this paper. It was found[16] that the RNA preparations closely resemble coiling synthetic polyelectrolytes[17–19] in their viscosity behavior, quite unlike DNA[20–23]. It appears from the viscosity pattern that unlike DNA, RNA consists of single contractile coils. This working hypothesis is supported by birefringence of flow measurements, and by the absence of irreversible changes in the potentiometric titration curves.

EXPERIMENTAL

Materials

The quality of the phenol was found to be important. Analytical grade phenol from several sources was found to contain small amounts of acid and to give very poor yields of RNA. Some batches were contaminated with traces of heavy metals and other impurities, which caused considerable degradation of the RNA. As a precaution, the phenol was therefore purified by distillation under reduced pressure.

ADP and Tris were obtained from Sigma Chemical Company. Crystalline lysozyme, pancreatic RNAase, and bovine plasma albumin were Armour and Company products. ^{32}P was purchased from the Radiochemical Center. *E. coli* polyribonucleotide phosphorylase was prepared according to the method of LITTAUER AND KORNBERG[24,25] and purified to the first ethanol step.

Analytical methods

Protein, nitrogen and phosphorus determination. Proteins were determined by the phenol method of LOWRY *et al.*[26]; crystalline bovine plasma albumin was used as the standard. For the determination of proteins in the presence of phenol, samples were precipitated with 3 vol. of 96 % ethanol containing 2 % K-acetate; after 20 min at 0° the precipitate was centrifuged in the cold and washed three times with 3.0 ml

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portions of cold 1 % PCA. The precipitate was dissolved in 0.6 ml of 0.1 *M* NaOH which contained 2 % Na_2CO_3, and protein was determined. This method was compared with the modification of the microbiuret method of ZAMENHOF AND CHARGAFF[27], and found to give identical values. Nitrogen was determined by the Dumas method and organic phosphorus according to KING[28].

Polysaccharide determination. For the determination of small amounts of polysaccharide contamination in the RNA, the following method was used: the RNA was hydrolyzed with NaOH and the resulting ribonucleotides removed by dialysis. The remaining polysaccharides were hydrolyzed with acid. The resulting monosaccharides were then determined as reducing sugar, by the ferricyanide method of PARK AND JOHNSON[29], glucose serving as standard. RNA was hydrolyzed in 0.5 *N* NaOH at 30° for 18 h, neutralized with HCl, and dialyzed for 48 h against 2 l of NaCl (1 *M*), and 2 l of cold water. Concentrated HCl was added to a final concentration of 0.6 *N*, and the polysaccharides were hydrolyzed by heating for 2 h in a boiling water bath. The solution was neutralized and aliquots were removed for reducing sugar determinations. In addition, polysaccharides were estimated by the detection of glucose in the HCl hydrolyzate after paper chromatography. A portion (1.0 ml) of the non-neutralized acid hydrolyzate was concentrated *in vacuo* and subjected to paper chromatography with phenol saturated with water, and developed with aniline phosphate reagent[30].

RNA and DNA determination. RNA was estimated by the orcinol method of DRURY[31] and DNA was determined with diphenylamine, as descibed by VOLKIN AND COHEN[32]. For the determination of nucleic acids in the presence of sucrose and proteins, the following procedure was used. To 1.0 ml-samples, 0.1 ml $MgCl_2$ (1 *M*), and 3.0 ml ethanol containing 2 % potassium acetate were added. After 30 min at 0°, the precipitate was centrifuged in the cold, and washed three times with 4.0 ml portions of cold PCA (0.2 *M*). The nucleic acids were then extracted from the precipitate with 2.0 ml PCA (0.5 *M*) at 70° for 20 min. The residue was centrifuged and aliquots were removed from the supernatant for nucleic acid determinations.

Nucleotide composition of RNA. RNA was hydrolyzed to its mononucleotides by dissolving it in NaOH at pH 13.0–13.5, and maintained at 30° for 18 h. The ribonucleotides thus obtained were separated by paper chromatography in isobutyric acid–0.5 *M* ammonia (5:3) and quantitatively determined by ultraviolet spectrophotometry. In each set of determinations, six portions of the hydrolyzate were subjected to chromatography[33,34].

Physical methods

Sedimentation in the ultracentrifuge. Sedimentation studies were performed on 0.3 to 0.6 % solutions of RNA in 0.2 *M* NaCl which contained 0.05 *M* phosphate buffer pH 6.8 (VOLKIN AND CARTER[13]). The sedimentation constants (*S*) were determined in the Spinco Ultracentrifuge model E, equipped with a Schlieren optical system; the values of *S* are expressed in Svedberg units (10^{13} sec). For the determination of the sedimentation constants, photographs were taken at 5 min intervals after maximum centrifugal speed was reached. The measurements were taken between 42,000 and 52,000 rev./min, and rotor temperature varied between 20 and 22° during the runs. We are grateful to Dr. M. GEHATYA and his staff for the sedimentation measurements.

Light scattering. These measurements were performed at 4360 Å and 20° with a Bryce-Phoenix type light-scattering photometer. Solutions were obtained by dissolving

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the polymer in 10^{-3} *M* NaCl in the cold, and subsequent addition of concentrated NaCl solution to a final concentration of 0.2 *M*. The solutions were clarified by centrifugation for 1 h at 10,000 *g*. Concentrations were based on phosphorus determinations, and expressed in g RNA/ml. We are grateful to Mr. Z. ALEXANDROWITCH for the light-scattering measurements.

Viscosity measurements. RNA solutions in water and in dilute NaCl solutions (below 10^{-3} *M* NaCl) were measured at 25° at low rates of shear ($G < 1$ sec^{-1}) in a Couette viscometer with electrostatic restoring torque[35]. Measurements at NaCl concentrations higher than 10^{-3} *M* were performed in a modified Ostwald-Fenske capillary viscometer standardized according to ASTM D445 (Series 50, $r = 0.0215$ cm, efflux time for 10 ml water = 245.3 sec at 25°, Kroepelin average rate of shear (for water $G = 1100$ sec^{-1})). RNA preparations dissolved in 10^{-3} *M* NaCl in about half an hour in the cold, without stirring, whereas solution in 10^{-1} *M* NaCl was very slow. Solutions (except those required for measurements in pure water) were always made up in 10^{-3} *M* NaCl, and brought to the proper ionic strength by dilution or by addition of concentrated NaCl. All solutions were found to be stable and the viscosity was unchanged over a few hours at 25°. Water used in this work was brought to pH 6.8 by bubbling of pure nitrogen and had a specific resistivity of $\sim 10^{-6}$ ohms.

Birefringence of flow. Measurements were performed at about 20° with an apparatus of the type described by EDSALL *et al.*[36].

Potentiometric titration. pH measurements were performed with a Beckman model G vacuum tube potentiometer and a glass, calomel-saturated KCl electrode system, accurate to ± 0.02 pH units. The instrument was standardized at 20° with 0.05 *M* potassium hydrogen phthalate (pH = 4.01) and 0.05 *M* sodium borate (pH = 9.18). Titrations were performed under nitrogen in 0.1 *M* NaCl with 0.1 *M* HCl and NaOH respectively. The salt concentration thus did not change during repeated forward and back titrations. A water correction was applied below pH 5 and above pH 9.

Ultraviolet absorption. Curve were determined at 20° with a Cary recording spectrophotometer. Individual absorption measurements at 260 mμ were made with a Beckman Model DU spectrophotometer.

Growth of cells

E. coli strain B was grown in a medium (pH 6.8 to 7.0) containing 1% yeast extract (Difco, dehydrated), 1% glucose, 2.18% K_2HPO_4 and 1.7% KH_2PO_4. Glucose was autoclaved separately[25]. For the preparation of inocula a small amount of surface growth from a 4–5 h culture grown on nutrient agar was suspended in sterile 0.9% NaCl solution to an optical density of 60 on the Klett-Summerson photoelectric colorimeter, using a red filter No. 66; 0.3 ml of this suspension were inoculated into a 4-l Erlenmeyer flask containing 1000 ml of medium. Cultures were incubated for 14 h at 37° with vigorous forced aeration until just the end of the logarithmic growth phase (optical density of 300 to 340). It was found that these growth conditions had to be followed very closely in order for stable protoplasts to be obtained. Cells harvested at the middle of the logarithmic growth phase or very old cells were found to give poor protoplasts, which lysed easily. The cells were harvested in a Sharples super centrifuge (5 to 6 g of wet cells) and washed twice with chilled Tris buffer (0.05 *M*, pH 9.0).

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Preparation of protoplasts

The preparation of protoplasts at pH 9.0 by the lysozyme method of ZINDER AND ARNDT[37] was found usually to give fragile preparations which lysed rapidly upon centrifugation or chilling. Neutralization of the alkaline suspension to pH 7.4, after the incubation with lysozyme, improved the stability somewhat, but some lysis still occurred during centrifugation.

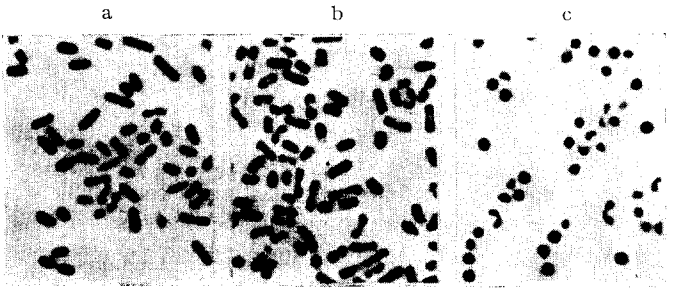

Fig. 1. Phase contrast microphotograph of wet mount of *Escherichia coli* (magnification 870 ×). (a) Cells, no lysozyme added. (b) "protoplasts" after 10 min incubation with lysozyme at 4°. (c) Protoplasts, after 10 min incubation with lysozyme at 37°.

It was found that incubation of the cells with lysozyme at 4° rather than 37°, resulted in rod-like "protoplasts" (Fig. 1b), which were stable enough to withstand several washings and centrifugations in the cold. When suspended in water, over 90 % of the "protoplasts" lysed within 10 min. These "protoplasts" had lost almost all of their cell wall mucopolysaccharides, but apparently retained some cell wall structure which kept them from assuming a spherical form (Fig. 1c) while the protoplasm usually was retracted to one or both ends of the cell. The following modification of the ZINDER AND ARNDT[37] procedure was finally adopted: the washed cells (10 g wet weight) were suspended in Tris buffer (0.05 *M*, pH 9.0), to a final volume of 40 ml, and incubated for 3 min at 37°. The temperature of the cell suspension was lowered to 4° and the following cold solutions were added with stirring: 20 ml sucrose (2 *M*), 12 ml water and 8 ml of lysozyme (2 mg/ml). After 10 min of incubation at 4°, 4 ml of Tris buffer (1 *M*, pH 7.4) were added. The "protoplasts" suspension was centrifuged for 6 min at 5,000 *g*, in a cold Servall centrifuge, and washed free of degraded cell wall polysaccharides and lysozyme, with two 30 ml portions of cold sucrose (1 *M*).

The progress of the lysozyme action was measured by comparing the degree of lysis of a water dilution of the suspension with that of a Tris buffer dilution; 0.2 ml aliquots were removed, and diluted with 5.0 ml water or 5.0 ml Tris buffer (1 *M*, pH 7.4) and the changes in the optical density were observed after 10 min at room temperature.

Isolation of RNA from "protoplasts"

The method consisted in: (a) removal of protein and DNA by phenol extraction, and (b) precipitation of RNA by ethanol or $(NH_4)_2SO_4$. The washed "protoplasts" (corresponding to 10 g weight cells) were lysed by suspending them in cold Versene (10^{-4} *M*, pH 8.0) to a final volume of 80 ml. For rapid suspension, use was made of a glass pestle whose diameter closely fitted that of the centrifuge tube. The cold viscous lysate was stirred while an equal volume of 90 % phenol was added quickly. The whole

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operation was completed within 5 min. The mixture was warmed to 20° and stirring was continued for 1 h at this temperature. The mixture was chilled and centrifuged immediately in polyethylene tubes in a cold Servall centrifuge for 3 min at 10,000 *g*. At the end of the centrifugation two phases separated: a lower phenol phase and an upper aqueous phase, containing the RNA. The turbid aqueous phase was decanted into a separatory funnel. After 5 min some additional phenol separated and was added to the remaining phenol phase. The phenol phase was mixed with 40 ml of Versene (10^{-4} *M*, pH 8.0) and, after 5 min in the cold the aqueous phase was removed by centrifugation. The aqueous solutions containing the RNA were now combined and centrifuged for 20 min, and the small quantities of phenol which separated were discarded.

Remarks. The washing of the protoplasts with sucrose was necessary in order to remove degraded polysaccharides, lysozyme and Tris buffer. The isolation of RNA in the presence of Tris buffer, particularly at pH 9.0, resulted in the extraction of DNA in the aqueous phase, while in the presence of sucrose the DNA remained in the phenol phase.

Ethanol precipitation. The RNA was precipitated from the cold aqueous solution by addition of 2 vol. of cold 96 % ethanol, containing 2 % K-acetate. After 30 min at 4° the precipitate was removed by centrifugation for 10 min at 10,000 *g*, washed with cold 75 % ethanol containing 2 % K-acetate and with 75 % ethanol respectively. The precipitate was dissolved in 25 ml of Tris buffer (0.01 *M*, pH 7.4) and small amounts of insoluble proteins were removed by centrifugation for 20 min at 10,000 *g*. The clear solution was dialyzed for 24 h against two 2-l changes of NaCl (10^{-3} *M*) at 4°. The slightly turbid solution was clarified by centrifugation for 20 min at 10,000 *g*, and then lyophilized. The yield of RNA amounted to 93–107 mg and represented 81–93 % of the initial RNA.

Ammonium sulfate precipitation. Precipitation of RNA from the aqueous solution could also be achieved with $(NH_4)_2SO_4$; 0.365 g of this salt per ml were added to the cold aqueous solution, and after 10 min at 4° the solution was centrifuged for 10 min at 10,000 *g*. The precipitate usually floated on top of the solution and was removed by careful decantation. It was dissolved in 25 ml of Tris buffer (0.01 *M*, pH 7.4) and small amounts of insoluble proteins were removed by centrifugation for 20 min at 10,000 *g*. The clear solution was dialyzed for 36 h against three 2-l changes of NaCl (10^{-3} *M*) at 4°. The slightly turbid solution was clarified by centrifugation for 20 min at 10,000 *g*, and then lyophilized. The yield of RNA amounted to 79–101 mg and represented 69–88 % of the initial RNA.

To remove heavy metal contamination (see results) the dialysis bags before use were soaked for several days in cold Versene solution (0.01 *M*, pH 8.0), which was changed several times. The bags were then washed with water.

RESULTS AND DISCUSSION

Experiments relating to the preparative procedure

Inactivation of crude E. coli *ribonuclease by treatment with phenol and water*

KIRBY[4] has shown that pancreatic RNAase is inactivated by incubation for one h at room temperature with phenol. It was important to determine whether *E. coli*

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RNAase was also inactivated at this temperature, in order to insure that the RNA was not degraded during the isolation procedure. To test this, *E. coli* RNAase was prepared in the following way: crude *E. coli* extract, containing RNAase, was obtained by the lysis of "protoplasts". To remove the nucleic acids the extract was incubated at 37° until the degradation of the nucleic acids came to a stop. The remaining material contained enzymes capable of hydrolysing RNA and DNA.

E. coli "protoplasts" (corresponding to 10 g wet weight cells), were lysed by suspension in water to a final volume of 128 ml. To the lysate the following solutions were added: 6.4 ml of Tris buffer (1 *M*, pH 7.4), 6.4 ml of $MgCl_2$ (0.1 *M*) and 19.2 ml phosphate buffer (1 *M*, pH 7.4). The viscous, turbid solution was incubated for 7 h at 37°, at which stage most of the RNA and DNA of the lysate was degraded. The turbid solution was dialyzed for 18 h against one l of NaCl (1 *M*), then for an additional 24 h against two 2-l changes of Tris buffer (0.01 *M*, pH 8.0). The turbid solution (185 ml) was centrifuged for 20 min at 10,000 *g* and the precipitate was discarded. The extract contained 0.18 mg/ml RNA and 0.01 mg/ml DNA. These amounts of nucleic acids correspond to 12.0 and 3.6 % of the initial RNA and DNA content of the original extract. Aliquots (5.0 ml) of crude *E. coli* RNAase were stirred with 5.0 ml 90 % phenol for 1 h, at 0 or 20°. The mixture was centrifuged, the phenol phase was removed and the aqueous phase phase extracted nine times with peroxide-free ether, to remove residual phenol. The ether was removed by bubbling nitrogen through the aqueous solution, and the volume made up to 5.0 ml. For RNAase assay, the incubation mixture (2.0 ml) contained 0.2 ml of Tris buffer (1 *M*, pH 7.4), 0.1 ml of NaCl

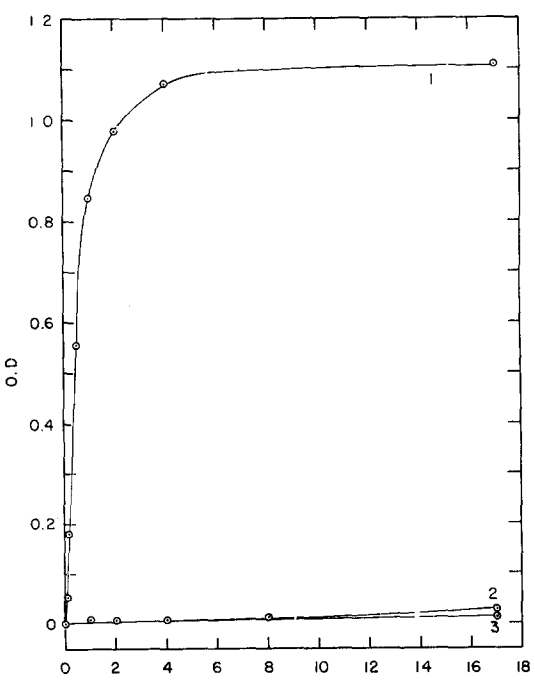

Fig. 2. Inactivation of *E. coli* RNAase by treatment with phenol and water. 1. Untreated RNAase. 2. RNAase treated with phenol at 0°. 3. RNAase treated with phenol at 20°.

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(5 *M*), 0.2 ml RNA (15 mg/ml) and 1.0 ml of treated or untreated RNAase. Incubation temperature was 37°. Aliquots (0.2 ml) were removed and chilled, 4.0 ml of cold PCA (0.13 *M*) were then added, after 10 min in the cold; the precipitate was centrifuged and the acid-soluble ultraviolet-absorbing material was measured as the difference in absorption at 260 and 290 mμ. From Fig. 2 it can be seen that treatment with phenol at 20° completely inactivated the RNAase, while at 0° some residual activity remained. Chromatography with isopropanol–ammonia[38] revealed an ultraviolet-absorbing spot corresponding in R_F to uridylic and cytidylic acids.

Influence of temperature on extraction of RNA from E. coli *cells or "protoplasts"*

Attempts to extract pure RNA from whole cells, in good yields, by modification of the method of WESTPHAL *et al.*[2] were not successful. *E. coli* cells were suspended in 80 ml water and were mixed with an equal volume of 90% phenol for 60 min at the temperatures indicated below. The suspension was centrifuged at 0° and the top aqueous layer removed. The remaining phenol phase was extracted once more with 40 ml cold water and the phases separated by centrifugation. RNA was precipitated from the pooled aqueous extracts by addition of two vol. of 96% ethanol containing 2% K-acetate. The precipitate was washed twice with cold 70% ethanol, dissolved in water and lyophilized. The resulting RNA was analyzed for its protein, polysaccharide and DNA content.

It was found that treatment of *E. coli* cells with phenol at 68° gave only a small quantity of RNA (4 to 12% of the initial RNA content); in addition, these preparations were heavily contaminated with polysaccharides (40 to 50%), as was also found by WESTPHAL *et al.*[2]. Attempts to extract RNA at 0 or 20° did not increase the yield, but in addition to polysaccharides, these preparations were also contaminated with proteins (10 and 4% respectively of the RNA content).

TABLE I

RNA ISOLATED FROM *E. coli* CELLS OR "PROTOPLASTS" *

Source	*Temperature of treatment with phenol* °C	*Yield of RNA* %	*Concentration in RNA*		
			Protein %	*Polysaccharide* %	*DNA* %
Cells	0	3.4	10.0	40.0	< 0.1
	20	10.0	4.0	50.0	< 0.1
	68	12.0	< 0.1	50.0	< 0.1
"Protoplasts"	0	67.0	4.6	0.8	< 0.1
	20	93.0	0.5	0.8	< 0.1
	68	12.0	12.0	—	0.7

* RNA precipitated with ethanol.

The use of bacterial "protoplasts" obviated the difficulties encountered in the attempts to isolate RNA from whole cells. The "protoplasts" were lysed by suspension in water and treated with phenol, and the RNA was isolated and analysed as described above. As seen from Table I, treatment of "protoplast" suspension with phenol gave high yields of RNA with very little polysaccharide impurities. The latter were probably removed by the lysozyme action. The use of phenol at 20° resulted in RNA

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preparations with low protein content, as compared with preparations obtained at 0° where the proportion of protein was higher and the RNA yield somewhat lower. The preparations contained only small amounts of polysaccharides (0.8 %) and DNA could not be detected (less than 0.1 %). Poor yields were obtained when the temperature was raised to 68°, and in addition to protein, these preparations contained small amounts of DNA.

Influence of Versene on the properties of RNA

When RNA was isolated by ethanol precipitation, its viscosity decreased considerably after dialysis. It was thought that this drop in viscosity might be due to the presence of traces of heavy metals in the dialysis bags. When the bags were washed thoroughly with Versene (0.01 *M*, pH 8.0), RNA solutions could be dialyzed without decrease in viscosity.

This led us to include Versene during the entire isolation procedure of RNA. The following experiments show that addition of Versene improved the quality of the final product. "Protoplasts" were lysed with either water or dilute Versene solution (10^{-4} *M*). The solution was then treated with phenol, and RNA was isolated by ethanol precipitation. Table II shows that in the absence of Versene the viscosity of the isolated RNA was lower; the effect was more pronounced when RNA was prepared at 20°. The sedimentation pattern showed a corresponding change. While all the RNA preparations revealed three boundaries in the ultracentrifuge (see below), in the absence of Versene the quantity of the heavier component ($S = 19.2$) decreased and the amounts of the lighter components increased (Table II).

TABLE II

EFFECT OF TEMPERATURE AND VERSENE ON THE PROPERTIES OF ISOLATED RNA

Temperature of treatment with phenol	*Versene*	*Viscosity* η_{sp}/c*	*Sedimentation peak number***		
			1	*2*	*3*
°C	*M*	*ml/g*	% *RNA*		
0	10^{-4}	1640	19	53	28
0	—	1400	20	66	14
20	10^{-4}	1490	21	46	33
20	—	707	32	60	8

* Viscosity measured in the presence of $5 \cdot 10^{-4}$ *M* NaCl, 10^{-4} g/ml RNA.
** Sedimentation constants of peak 1, 2 and 3 were 3.2, 14.2 and 19.2 respectively.

Ribonuclease activity in the RNA preparations

It was recently shown by ELSON[39], that a nucleoprotein which was isolated from *E. coli*, appeared to contain a latent ribonuclease. The RNA of the nucleoprotein remained intact at 0° unless the protein moiety was liberated by urea treatment at 0° [40]. When so liberated RNA was hydrolyzed rapidly. It was shown above (Table I) that the RNA prepared by treatment with phenol at 0° contained small quantities of protein (2 to 5 %), as compared with 0.5–0.8 % protein when the RNA was prepared at 20°. It was therefore important to decide whether the small protein contamination accompanying the RNA possessed some RNAase activity. RNA prepared by phenol treatment at 0° or 20°, and isolated by $(NH_4)_2SO_4$ precipitation, was incubated with

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TABLE III

RIBONUCLEASE ACTIVITY IN RNA PREPARATIONS

Preparation	Urea concentration M	14 hours		62 hours	
		Acid-soluble O.D.	Degradation %	Acid-soluble O.D.	Degradation %
RNA prepared with phenol at 20°	4.0	0.001	0.1	0.024	2.0
	0.0	0.000	0.0	0.000	0.0
RNA prepared with phenol at 0°	4.0	0.143	12.0	0.278	23.4
	0.0	0.019	1.6	0.178	15.0

The incubation mixture (2.1 ml) contained 1.0 ml RNA (1.6 mg/ml), and 0.1 ml NaCl (5 *M*). It was incubated at 37° with or without urea (4 *M*). 0.5 ml aliquots were removed and chilled, 3.5 ml of cold PCA (0.13 *M*) were then added; after 10 min in the cold, the precipitate was centrifuged and the acid-soluble ultraviolet-absorbing material was measured as the difference in absorption at 260 and 290 mμ.

or without 4 *M* urea, and the appearance of acid-soluble, ultraviolet-absorbing material at 37° was measured. With RNA prepared with phenol at 20°, no significant quantity of acid-soluble material could be detected, even after prolonged incubation periods (Table III). After 62 h of incubation with or without urea, 2.0 and 0.0 % of acid-soluble material appeared, respectively; with RNA prepared at 0° there was a small increase in acid-soluble material, amounting to 23.4 and 15 % respectively. The identity of this activity was not further studied. When the RNA preparations were incubated at 2° for 62 h, acid-soluble material did not appear. The slight activity noted with RNA prepared by treatment with phenol at 0°, was extremely small, as compared with the activity of isolated ribonucleoprotein in which the RNA was degraded after several hours of incubation in the presence of urea, and similar salt concentrations even at 2° *.

It was concluded that isolation of RNA at 20° gave a preparation superior with respect to purity and stability, while RNA isolated at 0° perhaps still contained a trace of RNAase activity.

Characterization and physical properties

From the above experiments the optimal conditions for the isolation of a high molecular weight RNA could be determined. The final procedure adopted is given in METHODS and the composition, fractionation and physical properties are discussed below.

Nucleotide composition of the RNA

The average nucleotide composition of RNA isolated by $(NH_4)_2SO_4$ precipitation (see below) is shown in Table IV. The figures differ only very slightly from those of ELSON AND CHARGAFF[41] and LOMBARD AND CHARGAFF[42]. The uridylic acid value was found to be a little higher and the cytidylic acid value slightly lower than given in these publications. In accordance with these workers the ratio of 6-amino to 6-keto nucleotides was found to be near unity.

* Dr. D. ELSON, personal communication.

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TABLE IV

ANALYSIS OF RNA PRECIPITATED WITH $(NH_4)_2SO_4$

Phosphorus, % dry weight	8.05
Nitrogen, % dry weight	14.08
Phosphorus/Nitrogen (weight ratio)	1.75
Adenylic acid, moles per 100 moles*	26.3
Cytidylic acid, moles per 100 moles*	22.8
Guanylic acid, moles per 100 moles*	28.9
Uridylic acid, moles per 100 moles*	22.0
6 – Am/6 – K, molar ratio**	0.965
$\varepsilon(P)_{260\,m\mu}$, 1.0 *M* phosphate pH 7.1	7,290
$\varepsilon(P)_{260\,m\mu}$, after alkaline hydrolysis, 1.0 *M* phosphate pH 7.1	11,600
$\varepsilon(P)_{260\,m\mu}$, after RNAase action, 1.0 *M* phosphate pH 7.1	9,560
$\varepsilon(P)_{260\,m\mu}$, after polyribonucleotide phosphorylase action, 1.0 *M* phosphate pH 7.1	11,700

* The values are based on analysis of five preparations; the recovery was 98.2 % based on ultraviolet absorption.

** The ratio of the molar sum of 6-amino nucleotide (adenylic and cytidylic acids), to that of the 6-Keto nucleotide (guanylic and uridylic acids).

Isolation and molecular weight of RNA preparations

RNA preparations isolated by ethanol precipitation gave three boundaries in the ultracentrifuge. The sedimentation constants S of the two higher peaks varied in different preparations from 19 to 24 and 14 to 16; these two peaks amounted to 80–90 % of the RNA. The lighter peak with $S = 3.2$ was broad and polydisperse. The sedimentation constants were not extrapolated to zero concentrations of the polymer. Attempts to separate the two heavier components from the lightest one by $(NH_4)_2SO_4$ precipitation failed unless the solution was saturated with phenol. Under these conditions a nearly complete separation was achieved at 0.57 $(NH_4)_2SO_4$ saturation (Table V). This heavier fraction was used for the physical measurements. In practice, the RNA was precipitated from the phenol-saturated aqueous extract of the "protoplast" lysate.

TABLE V

PRECIPITATION OF RNA WITH ETHANOL AND $(NH_4)_2SO_4$

Method of isolation	*Viscosity* η_{sp}/c* *ml/g*	*Sedimentation peak number***		
		1	*2*	*3*
		% *RNA*		
Ethanol precipitate	780	19	53	28
$(NH_4)_2SO_4$ precipitate	1,230	trace	65	35
$(NH_4)_2SO_4$ supernatant	34	100	0.0	0.0

* Viscosity measurement in the presence of $5 \cdot 10^{-4}$ *M* NaCl, 10^{-4} g/ml RNA.

** Sedimentation constants of peak 1, 2 and 3 were 3.2, 14.2, 19.2 respectively.

Preliminary light-scattering measurements with the polymer isolated by precipitation with $(NH_4)_2SO_4$ gave a molecular weight (mol. wt.) of $(1.5 \pm 0.4) \cdot 10^6$; the value of the refractive index increment $dn/dc = 0.194$ as given by HOPKINS AND SINSHEIMER[8] for TMV-RNA at 4360 Å was used. In view of the heterogeneous

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character of this material, no quantitative significance should be attached to this mol. wt. average as obtained by the light-scattering measurements. It suffices to conclude, for the sake of subsequent discussion, that both the sedimentation constants and light-scattering results indicate a highly polymerized material. A rough estimate of the mol. wt. may also be obtained by a combination of viscosity data in 0.2 *M* NaCl (the limiting viscosity number $[\eta] = 36.2$ ml/g at 25°) and the value of the sedimentation constants, using the equation of FLORY[43]. The higher peak ($S = 19$ to 24) gives by this procedure a mol. wt. of about 6 to $8 \cdot 10^5$, whereas the second peak ($S = 14$ to 16) leads to a mol. wt. about 3.5 to $4.5 \cdot 10^5$.

Viscosity measurements

Rate of shear dependence. The viscosity of solutions of RNA in pure water was strongly shear-dependent. The values of the viscosity numbers (specific viscosity divided by concentration) η_{sp}/c measured at low rates of shear G were about twice as

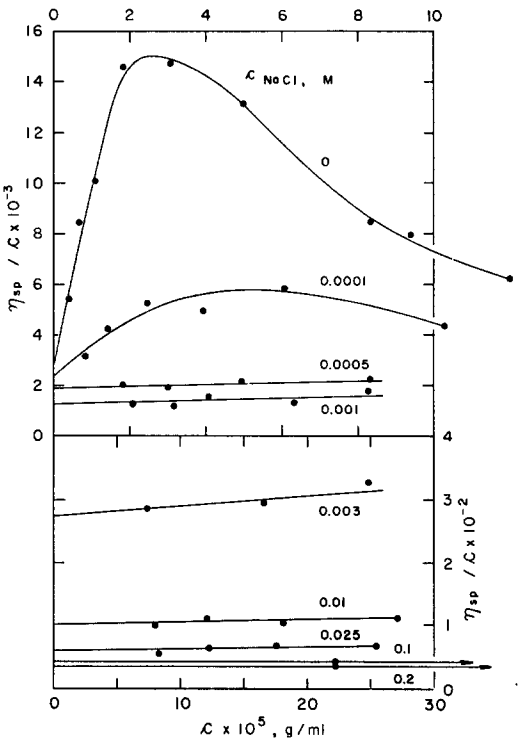

Fig. 3. Viscosity numbers of RNA solutions at various NaCl concentrations at 25°. NaCl concentrations are marked on each curve.

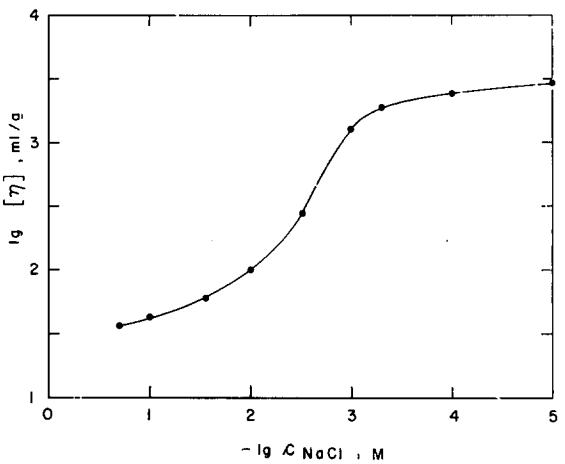

Fig. 4. Limiting viscosity numbers of RNA solutions as a function of NaCl concentration.

large as those determined at the higher rates of shear in the capillary viscometer. The dependence of η_{sp}/c upon G persisted in the extrapolation to zero concentration (limiting viscosity numbers $[\eta]$), and is thus not due to structural factors in the solutions. Upon addition of NaCl, η_{sp}/c decreased and the shear dependence gradually disappeared. At a NaCl concentration of 10^{-3} *M* the measurements at low rates of shear (determined in the rotation viscometer) agreed within experimental error with the data obtained in the capillary viscometer.

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Dependence on concentration and ionic strength. The dependence of the viscosity numbers η_{sp}/c upon concentration of RNA is represented in Fig. 3. It is seen that η_{sp}/c of aqueous solutions at first increases with decrease of concentration; the curve reaches a maximum at a low concentration of the polymer and then rapidly decreases with further decrease in concentration. In very dilute salt solutions (10^{-4} M NaCl) the maximum in the curve is very much depressed, and it completely disappears at NaCl concentration as low as $5 \cdot 10^{-4}$ M. At higher concentrations of NaCl the extrapolation plots are almost linear in the concentration range investigated. The viscosity in 0.1 M NaCl ($[\eta] = 42.5$) and 0.2 M NaCl ($[\eta] = 36.2$) is concentration-independent up to $c = 10^{-3}$ g/ml.

The viscosity numbers η_{sp}/c in Fig. 3 were extrapolated to zero concentration of RNA to give the limiting viscosity numbers $[\eta]$, which are shown in Fig. 4 as a function of NaCl concentration. It is seen that $[\eta]$ does not change appreciably up to about $5 \cdot 10^{-4}$ M NaCl. At higher NaCl concentration $[\eta]$ drops sharply and again levels off at around 0.1–0.2 M. The total change from very low values of NaCl concentration to the higher values amounts to about a hundred-fold decrease in $[\eta]$.

The two characteristic features of the viscosity curves just examined namely: (a) the large maxima in the η_{sp}/c curves at very low concentration of added electrolytes and (b) the pronounced decrease of $[\eta]$ with increase in NaCl concentration, are strongly reminiscent of the viscosity behavior of coiling synthetic polyelectrolytes[17–19].

This viscosity behavior of RNA strongly contrasts with that of DNA[20–23]. In aqueous solutions η_{sp}/c of DNA continually increases with concentration of the polymer and no maximum in the curve has been observed. The extrapolated values of $[\eta]$ shows little dependence upon the ionic strength up to the highest concentration of added electrolyte. The insensitivity of the shape of the DNA molecule to changes in ionic strength has been attributed to the stiffening influence of the proposed intertwined double helix structure[44]. It is believed that the molecular architecture due to the double helix prevents collapse of the DNA molecule when the electrostatic charges are screened off. While the double helix structure of DNA thus appears to be sufficiently stiff to prevent expansion and contraction with change in electrostatic interactions, a single-strandedp olynucleotide chain can be shown to be endowed with sufficient flexibility[45] to allow for stretching and contraction as a result of electrostatic forces.

It appears from the viscosity measurements that the RNA preparations investigated exist in solution in the form of flexible single strands. They are assumed to expand at low values of ionic strength, following electrostatic repulsion between the phosphate charges on the RNA back bone; at high values of ionic strength, the molecular chains are assumed to contract owing to electrostatic screening of the phosphate charges. If one assumes a hypothetical molecule of mol. wt. $= 10^6$ and applies to it FLORY's formula[43] relating end-to-end distance r to $[\eta]$ and mol. wt. in coiling polymers, one may calculate $r = 2500$ Å corresponding to $[\eta] = 3000$ in water and $r = 600$ Å corresponding to $[\eta] = 40$ in salt solutions. The contractile model for the RNA preparations is supported by the disappearance of birefringence of flow (see below) with increase in ionic strength, and by the fact that no irreversible changes in the potentiometric titration curve (see below) have been observed following repeated backward and forward titration to acid (pH 2.8) and alkaline (pH 10.8) conditions. However, no deduction as to the structure of RNA in the living cell can be

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made from the behavior of isolated preparations in solutions. It is interesting to note that FRANKLIN *et al.*[46] have recently shown from X-ray studies of TMV and turnip yellow mosaic virus that the RNA in the virus particle is in the form of a single strand, which conforms to the structure imposed on it by the virus protein; a similar structure was suggested by them for microsomal particules of rat liver and yeast.

Remarks. While the nucleic acid–protein bond in DNA nucleoprotein is assumed to be electrostatic, it has been suggested[47] that in the case of RNA nucleoproteins, hydrogen bonds are involved. Thus, while DNA may be isolated from the nucleoproteins by procedures involving electrolytes which do not effect hydrogen bonds, mild hydrogen bond breakers are required for the isolation of RNA. It was therefore necessary to show that reagents such as the solutions of phenol used in this work do not destroy possible structures in RNA similar to those exhibited by the double helix of DNA. Any reagent attacking this latter structure might also cause structural damage to RNA should the native RNA exist in a similar form. Therefore, solutions of high-molecular weight samples of trout sperm and calf thymus DNA* (mol. wt. $\sim 6 \cdot 10^6$, $[\eta] \sim 6000$ ml/g in 0.1 M NaCl) were shaken with saturated phenol, under the conditions used for the isolation of the RNA. The DNA was then isolated by ethanol precipitation. No decrease in the values of $[\eta]$ in 0.1 M NaCl was observed. Furthermore, BRAUN *et al.*[48] have shown that incubation of *Brucella abortus* cells for 48 h with 0.5 % phenol at 37° may be used to extract DNA still possessing specific transforming activity. While no full analogy with RNA may be claimed, it is gratifying to note that mild treatment with phenol does not lead to irreversible changes in the case of DNA.

We have also tested the possibility of the viscosity behavior of RNA being due to the presence of molecular aggregates. The viscosity in 10^{-3} M and 10^{-1} M NaCl was found to be unaffected by 4 M urea. Moreover the reversibility of the viscosity behavior with salt concentration was tested. A stock solution (10^{-2} g/ml) of RNA was made up in 10^{-3} M NaCl. To a part of this solution 5 M NaCl solution was added to a total of 0.1 M concentration. The original stock solution was diluted one hundred fold with 10^{-3} M NaCl, while the RNA solution in 0.1 M NaCl was diluted one hundred fold with pure water. The viscosity numbers η_{sp}/c of both dilute solutions were found to be identical, in agreement with the data plotted in Fig. 3. Also, when the stock solution was brought to pH 2.8 by addition of conc. HCl, and back to pH 7.0 by addition of an equivalent amount of conc. NaOH (and alternatively to pH 10.8 and back to pH 7.0) no specific viscosity changes were found following dilution to 10^{-3} M NaCl concentration. The viscosity at 10^{-3} M NaCl of a dilute (10^{-4} g/ml) RNA solution thus did not depend on the history of the solution either with respect to NaCl concentration or pH (within the given pH values).

Influence of Mg^{++} and phosphate ions on viscosity. Small amounts of Mg^{++} appreciably reduced viscosity of a solution of RNA in water. Thus when $MgCl_2$ concentration was increased from 0 to 10^{-5}, $3 \cdot 10^{-5}$, $6 \cdot 10^{-5}$, 10^{-4} and $2 \cdot 10^{-4}$ M, η_{sp}/c ($c = 12 \cdot 10^{-5}$ g/ml) dropped from 6400 to 4250, 2060, 480, 115 and 60, respectively. This decrease was considerably larger than at corresponding ionic strengths due to NaCl. Small amounts of phosphate also appreciably reduced η_{sp}/c. Thus in $5 \cdot 10^{-4}$ M K-phosphate, $\eta_{sp}/c = 110$ as compared with 6400 in water.

* We are grateful to Drs. P. DOTY AND R. VENDRELY for the samples of DNA.

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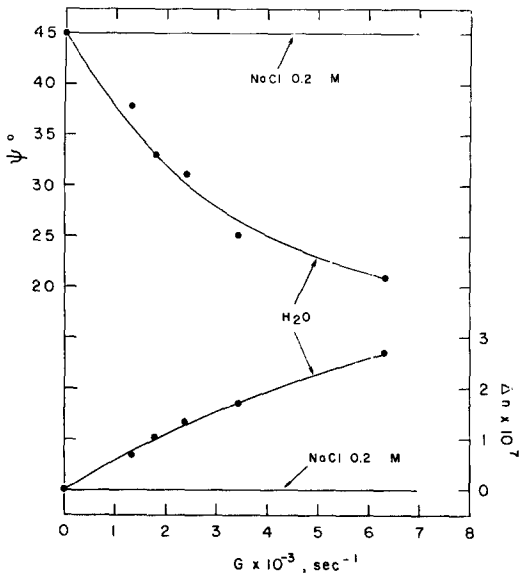

Fig. 5. Angle of orientation ψ and amount of birefringence Δn in RNA solution ^{c}RNA = $2.8 \cdot 10^{-3}$ g/ml.

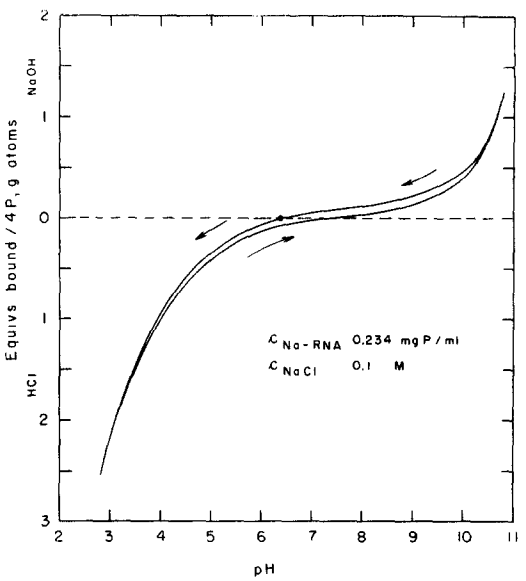

Fig. 6. Potentiometric titration curves of RNA.

Birefringence of flow

These measurements are summarized in Fig. 5. We have determined the extinction angle ψ (which is known[49] to depend only on the size and shape of the particles) and the birefringence Δn (which is related[49] to the size, shape and optical properties of the solute and solvent molecules) at various rates of shear G. The sign of birefringence in neutral aqueous solutions of RNA was found to be positive as opposed to that of DNA which shows[49] negative birefringence of flow. The positive birefringence observed for RNA may be compared with the measurements of FRANKLIN[50] who found a positive contribution to birefringence of flow in TMV suspensions, which disappeared upon removal of the RNA constituent from the virus particle. It is further interesting to note (particularly in view of the viscosity results), that the small birefringence exhibited by the RNA preparations in pure water solutions (Fig. 5) completely disappeared upon addition of salt (0.2 M NaCl). Under the same conditions no orientation was observed ($\psi = 45°$ at all rates of shear). In solution of DNA considerable birefringence and orientation persists at neutral pH even in the highest concentrations of NaCl (2 M) that have been investigated[49].

Potentiometric titration

A typical potentiometric titration curve of a RNA preparation in 0.1 M NaCl is given in Fig. 6. The solution before titration was dialyzed against CO_2-free water to pH 7.0 for 24 h; during dialysis pure nitrogen was bubbled through the equilibrating water. Upon addition of concentrated NaCl to a 0.1 M concentration, the pH decreased to 6.02. The complete titration curve between pH 2.8 and pH 10.8 is represented by a closed loop with relatively small hysteresis. There does not appear to exist an irre-

versible step in the titration curve as with DNA and a complete titration cycle brings the pH back to its original value. This is in line with an observation on RNA preparations isolated from *Aerobacter aerogenes*, reported by Cox *et al.*[51]. No hypothesis for the small, but significant, hysteresis loop in the titration curve is advanced.

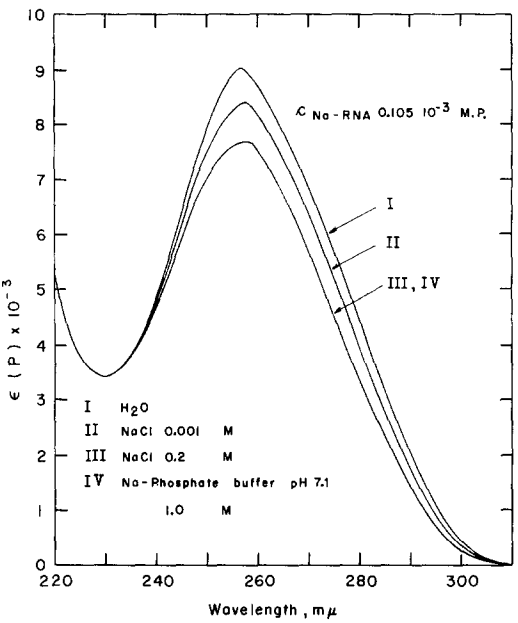

Fig. 7. Ultraviolet absorption spectra of RNA.

Ultraviolet absorption

Influence of salts. The ultraviolet spectra of the RNA preparations are shown in Fig. 7. The molar extinction coefficient[52] ε (P) at 260 mμ decreased from 8700 in H_2O to 8160 in 10^{-3} *M* NaCl to 7450 in 0.2 *M* NaCl. (7290 to 7450 for different preparations). The spectrum in 0.2 *M* NaCl was identical with that obtained in 1.0 *M* sodium phosphate buffer, pH 7.1. A decrease in ε(P) upon addition of phosphate buffer was also noted by REDDI[53] for TMV RNA. The maximum in the absorption curve shifted from 257.0 mμ in H_2O to 257.5 mμ in 10^{-3} *M* NaCl to 2.58.0 mμ in 0.2 *M* NaCl and in the phosphate buffer. For routine measurements of RNA, values in 1 *M* phosphate buffer should be preferred to those in distilled water.

Alkaline hydrolysis. After treatment of RNA with KOH (pH 13.5) the ε(P) value increased by about 56 to 59 % (Table IV); such a high increase has not been reported previously[12,14,54–56].

Enzymic degradation. After extensive pancreatic RNAase action, $\varepsilon(P)_{260}$ increased only by about 28 to 31 % for different preparations, which might be correlated with the inability of RNAase to degrade RNA completely, leaving behind a nondialysable limit polynucleotide ("core") with strong hyperchromic effect[53,55]. Phosphorolysis of RNA with polyribonucleotide phosphorylase[25] increased $\varepsilon(P)_{260}$ to the same extent as alkaline hydrolysis (Table IV, Fig. 8). Whether this high increase in ε(P) as compared with that obtained with pancreatic RNAase, is due to the more extensive degradation of the RNA or to the disruption of different bonds between the component nucleotide of RNA awaits further study.

The action of different concentrations of pancreatic RNAase on RNA was followed by parallel measurements of viscosity and optical density (Fig. 9). Of interest is the observation that at low enzyme concentrations the optical density remains constant though viscosity decreases rapidly. It thus appears that, at low enzyme concentration, changes in viscosity constitute a very sensitive criterion for the action of RNAase. Whether this marked drop in viscosity is due to enzyme–substrate interaction (and subsequent changes in configuration of the complex) or to decrease in mol. wt., or both, requires further study.

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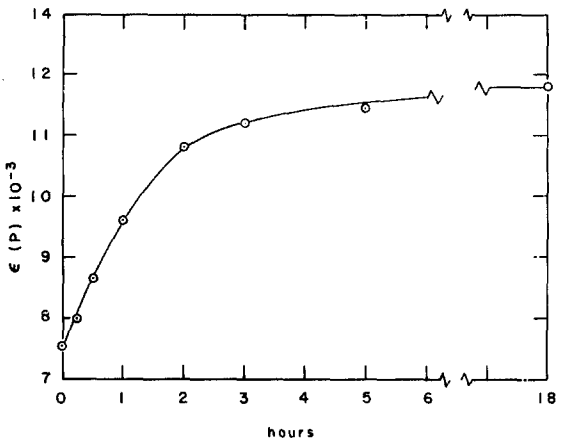

Fig. 8. Effect of polyribonucleotide phosphorylase on the absorption of RNA at 260 mμ. The incubation mixture (1.0ml) contained 0.20 ml of RNA solution (6.84 μM P/ml), 0.04 ml of Tris buffer (1.0 M, pH 8.0), 0.04 ml of $MgCl_2$ (0.1 M), 0.06 ml of K-phosphate buffer (1.0 M, pH 7.4) and 2.5 units of enzyme (Assay C[25]). The mixture was incubated at 25°. Aliquots (0.1 ml) were withdrawn and diluted with 4.0 ml of 1.0 M Na-phosphate buffer pH7.1 for optical density measurements at 260 mμ.

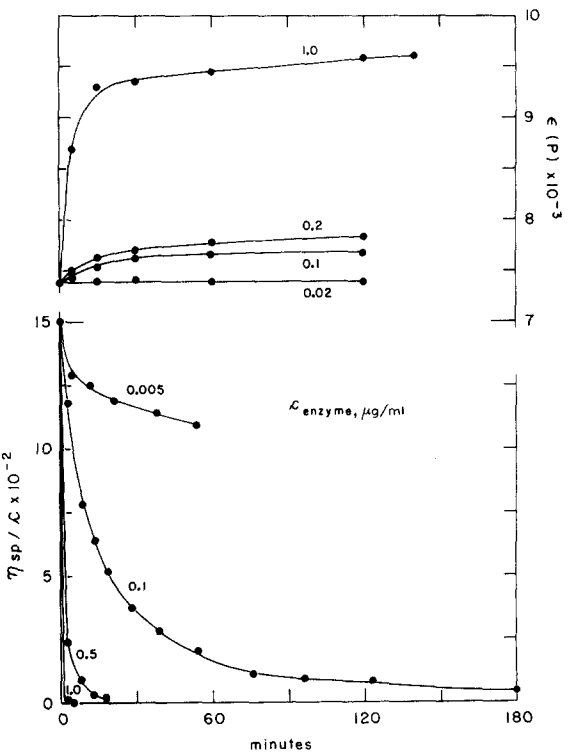

Fig. 9. Effect of pancreatic RNAase on the viscosity and on the absorption (at 260 mμ) of RNA. To the RNA solution (10^{-4} g/ml, 10^{-3} M NaCl) varying amounts of RNAase were added, incubated at 25° and the decrease in viscosity followed with time (Fig. 9, lower part). Aliquots (0.5 ml) were withdrawn from another portion of the same solution and diluted with 1.0 ml of 1.0 M Na-phosphate buffer pH 7.1 for optical density measurements at 260 mμ (Fig. 9, upper part). Enzyme concentrations (μg/ml) are marked on each curve.

Viscosity changes upon addition of polynucleotide phosphorylase were not studied since the enzyme acts only in the presence of magnesium and phosphate ions which (see above), by themselves, depress the viscosity markedly.

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