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Protein–Nucleic Acid Interaction. I. Nuclease-Resistant Polylysine–Ribonucleic Acid Complexes*

Herbert A. Sober, Stuart F. Schlossman,† Arieh Yaron,‡ Samuel A. Latt, and George W. Rushizky

ABSTRACT: Polylysines react on an equivalent basis with ribonucleic acid (RNA) to form insoluble complexes at low salt concentrations and neutral pH. Soluble complexes are formed, however, at low polylysine:RNA ratios.

Digestion by nonspecific ribonucleases results in the formation of a precipitate with a lysine:nucleotide ratio of 1. The "protected" nucleotide chain has essentially the same chain length as the polylysine of the initial complex and is susceptible to cleavage by the original enzyme. Protection specificity, *i.e.*, high Gp + Cp content of protected fragment, appears to be related to secondary structure and is eliminated by thermal denaturation. Implications of these data in terms of a model of protein–nucleic acid interaction are discussed.

Complexes of proteins and nucleic acids (nucleoproteins), which occur within the cell virtually wherever nucleic acids are found, may play a controlling role in processes of cell growth, differentiation, and replication. While it is known (Katchalsky, 1964) that interaction between oppositely charged polyelectrolytes produces complexes with altered properties, relatively little is known about the specific nature of the interaction, the mechanism by which these processes are effected, or whether sufficient specificity to satisfy the biological requirements can be achieved by such interaction.

This paper is concerned with the interaction between polylysines of relatively well-defined size (the protein model) and ribonucleic acid (RNA), and the effect of this interaction on the hydrolysis of RNA by nucleases. In common with similar studies with deoxyribonucleic acid (DNA) (Spitnik *et al.*, 1955) we have found that, at low salt concentrations (0.10 M) and neutral pH, the addition of polylysine to RNA in solution results in the formation of a precipitate as the two components approach charge equivalence. However, at low polylysine:RNA ratios, complexes of these polyelectrolytes are soluble and amenable to study.

The experimental approach used in our studies is to form these soluble polylysine–RNA complexes and to treat them with nuclease. During the course of digestion, short oligonucleotides and mononucleotides appear and a precipitate is formed which contains both polylysine and RNA material with a nucleotide:lysine ratio of 1. After isolation of the precipitated complex and dissociation and separation into its components, the RNA member of the complex is examined as to chain length, base composition, and susceptibility to nuclease action. The results obtained from these studies establish that the polylysine–RNA complexes contain nuclease-resistant RNA segments, that these protected RNA segments are of essentially the same chain length as the polylysine, and that under certain

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received August 22, 1966.* A preliminary report has appeared (Sober *et al.*, 1965).

† Present address: Department of Medicine, Harvard Medical School, Beth-Israel Hospital, Boston, Mass.

‡ Visiting Scientist. Present address: Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

conditions, the nucleotide composition of the protected segment differs from that of the parent molecule.

Materials and Methods

RNA. Yeast RNA was prepared by the hot detergent method of Crestfield *et al.* (1955); yeast soluble ribonucleic acid (s-RNA) was purchased from General Biochemicals. TMV-RNA was a gift from Dr. C. A. Knight, University of California, Berkeley, Calif. RNA from bacteriophage MS2 was prepared essentially as described by Strauss and Sinsheimer (1963) and taken to have an A_{260} of 25 for 1 mg/ml at neutral pH and low salt. The heat denaturation of MS2-RNA is described in the legend to Table III. The oligonucleotide hexamer fraction used as a marker was obtained from a ribonuclease T_1 (Sato and Egami, 1957) digest of yeast RNA by procedures reported previously (Rushizky *et al.*, 1964a).

Enzymes. Pancreatic ribonuclease (two times recrystallized from alcohol) was purchased from Worthington. Ribonuclease T_1 was prepared by the method of Rushizky and Sober (1962) and nonspecific ribonuclease from *B. cereus* was prepared according to the procedure of Rushizky *et al.* (1964b). Micrococcal nuclease (90% pure) was a gift from Drs. H. Taniuchi and C. B. Anfinsen, National Institutes of Health (Anfinsen *et al.*, 1963). Pronase (grade B) was purchased from Calbiochem. Separate alkaline phosphatase preparations from *Escherichia coli* containing 2000–3000 *p*-nitrophenylphosphate units/mg of protein (Koerner and Sinsheimer, 1957) were donated by Father Donald J. Plocke, Peter Bent Brigham Hospital, Boston, Mass., and Dr. Leon Heppel, National Institutes of Health (Neu and Heppel, 1965).

Adsorbents. Sephadex G-200 was obtained from Pharmacia. DEAE-cellulose (Whatman Floc DE-50, Lot 76–82, 1.0 mequiv of N/g, 100–230 mesh) was washed as described by Peterson and Sober (1956).

Polylysine hydrochloride of average chain length $\bar{n}$ = "100" was obtained from the Department of Biophysics, Weizmann Institute of Science, Israel. After partial hydrolysis of the long-chain polymer, individual oligolysine polymers of specific chain length[1] were isolated by chromatography on CM-cellulose (Yaron *et al.*, 1964). Spectrophotometric measurements were made in silica cells of 1-cm light path and expressed as absorbance (A).

Enzymatic Digestion of Polylysine–RNA Complexes. Most of the experiments reported here used soluble complexes with a lysyl:nucleotide residue ratio of 1:5 (1:10, w/w).

In a representative experiment with micrococcal nuclease, 10 mg of polylysine "30" was dissolved at room temperature in 5 ml of 0.01 M Tris·HCl, pH 8.5 (0.01 M in Tris), and added dropwise over a period of 30 min with stirring to a solution of 100 mg of yeast RNA in 5 ml of the same buffer. (The volume was doubled when MS2-RNA was used.) After the addition of 0.1 ml of 0.1 M $CaCl_2$ and 0.1 ml of nuclease (0.15 mg/ml), the solution was incubated at 39° for 16 hr. The insoluble material formed during digestion (polylysine-protected RNA fragment) was removed by centrifugation.

An aliquot (0.2 ml) of the supernatant solution was subjected to paper chromatography on Whatman No. 3 paper using a solvent of *n*-propyl alcohol–H_2O–concentrated NH_4OH (55:35:10, v/v). Enzymatic digestion of MS2-RNA was judged to be complete under these conditions since no ultraviolet-absorbing material remained at the origin of the chromatogram and only mono- and dinucleotides were present (Roberts *et al.*, 1962), accounting for all the optical density in the supernatant.

The precipitate (consisting of protected RNA segment and polylysine) was washed by resuspension in water followed by centrifugation, and dissolved in 10 ml of a solution 7 M in urea, 0.1 M in NH_4HCO_3, pH 8.6, and 0.01 M in EDTA. (EDTA was used to halt any further nuclease action since both micrococcal nuclease and the *Bacillus cereus* enzyme require Ca^{2+} for activity.)

This solution of solubilized polylysine and protected RNA fragment, which contained 19 mg of RNA material by spectrophotometry, was then adsorbed on a 4 × 20 cm (internal diameter × height) column packed with DEAE-cellulose (preequilibrated with starting buffer, 7 M urea–0.1 M NH_4HCO_3, pH 8.6). The column was washed with 1 l. of starting buffer and with water until the A_{230} of the effluent was 0.02 or less, indicating removal of polylysine, EDTA, and urea. About 17.5 mg of the RNA material was then recovered from the column by elution with 2 M NH_4HCO_3–concentrated NH_4OH (50:50). It was then diluted threefold with water, lyophilized, and stored over $CaCl_2$ in a desiccator at room temperature or as a frozen solution.

Similar experiments were performed with other nucleases. A nonspecific ribonuclease from *B. cereus* was employed at 20 units/mg RNA in 0.01 M Tris·HCl, pH 7.3–0.001 M $CaCl_2$ (Rushizky *et al.*, 1964b). Pancreatic and T_1 ribonucleases were used in 0.1 M Tris·HCl, pH 7.5, alone or in the presence of 0.001 M $CaCl_2$ or $MgCl_2$. Sufficient enzyme was used to ensure complete hydrolysis of the RNA.

Pronase[2] was used to remove polylysine from the insoluble polylysine-protected RNA complex as follows. MS2-RNA (100 mg) and polylysine "30" (10 mg) were treated with *B. cereus* nuclease as described above. The insoluble complex so obtained was washed with water and 0.001 M EDTA, pH 7.5. An aliquot was dissolved and the RNA fragment was isolated as above.

[1] Chain lengths of polylysine preparations cited in quotation marks are average chain lengths (n); those listed without quotation marks are individual oligomers or known mixtures in which the chain length distribution is restricted and defined.

[2] Commercial trypsin could not be used in this manner since it contained sufficient nuclease activity to hydrolyse RNA to completion.

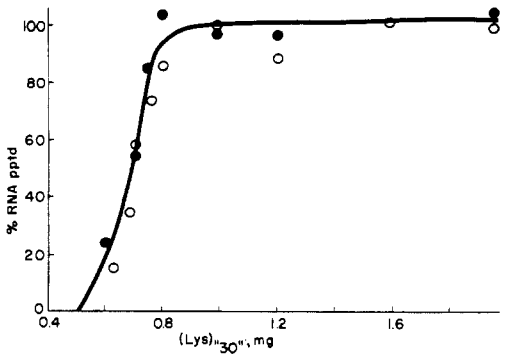

FIGURE 1: Insoluble polylysine–RNA complex. Details of reaction conditions are given in the text under Methods and Materials. Solid line: absorbance at 260 mμ. Open circles: phosphorus values. Closed circles: nitrogen values. All values have been normalized to conform with the scale of the ordinate.

Another aliquot of the precipitated complex was suspended in 0.02 M Tris·HCl–0.001 M EDTA, pH 7.5, and incubated with Pronase (enzyme:substrate, 1:50, w/w) until the complex dissolved (4 hr at 39°). Solutions obtained after Pronase action were then examined for RNA hydrolysis by paper chromatography, directly or after further exposure to *B. cereus* nuclease in 0.001 M $CaCl_2$.

Chain-Length Determinations and Hydrolysis Conditions. The micromethod of Ames and Dubin (1960) was used for the determination of total and terminal phosphate. Alkaline phosphatase digestions were performed with 400–2500 units/mg of RNA in 1.5-ml final volume (0.2 M Tris·HCl, pH 8.5, 1–2 mg of RNA, 3 or 17 hr at 39°). Conditions for complete digestion were established by using various concentrations of enzyme and RNA. Alkaline hydrolysis of RNA, with or without prior dephosphorylation, was in 1 N KOH at 23° for 24 hr. Digests were examined by paper chromatography as described above or by paper electrophoresis at pH 3.7 in 0.2 M Tris–acetate at 6 v/cm for 16 hr. The nucleoside:nucleotide ratio was obtained by paper chromatography.

N:P Ratios of Polylysine–RNA Complexes. Soluble complexes of yeast RNA and native or heated MS2-RNA were prepared with polylysine "30" and polylysine 21–25 and then digested with *B. cereus* ribonuclease as described above but in 0.1 M $NaHCO_3$, pH 7.9. The precipitate was washed twice by centrifugation and resuspension in water, and finally dissolved in 0.1 N KOH. Values for nitrogen (Perrin, 1953) and phosphorus (Ames and Dubin, 1960) of the RNA–polylysine complexes were then compared with those obtained separately from RNA and the corresponding polylysine preparations.

In addition, insoluble complexes of polylysine and RNA were prepared directly by the slow addition of various amounts of polylysine "30" (from 0.5 to 2.0 mg at 1 mg/ml in 0.01 M sodium citrate titrated to pH 7.0) to separate 2-ml aliquots of the same RNA solution (1 mg/ml of yeast RNA in 0.01 M sodium citrate, pH 7.0). The final volume was 5 ml. After mixing by inversion, the samples were allowed to equilibrate overnight and the precipitate was removed by centrifugation for 30 min in a 40 rotor at 20,000 rpm (Spinco Model L), extensively washed in the citrate solution, dissolved in 0.1 M NaOH, and analyzed for nitrogen (Perrin, 1953) and phosphorus (Boltz and Mellon, 1947).

Amino Acid Acceptor Activity of s-RNA Protected by Polylysine. A sample of protected s-RNA, resulting from a polylysine "30" protection experiment, was desalted on DEAE-cellulose and tested (Doctor *et al.*, 1963) for amino acid acceptor activity with [^{14}C]algal protein hydrolysate or [^{14}C]serine. The original s-RNA served as control. Original charging activities per milligram of s-RNA were 9200 cpm of [^{14}C]algal protein hydrolysate and 1.4 mμmoles of [^{14}C]serine, respectively.

Results

Insoluble Polylysine–RNA Complexes. The addition of increasing amounts of polylysine to RNA in solution, at low salt concentration and neutral pH, leads to the formation of a precipitate containing both polylysine and RNA. As shown in Figure 1, complete precipitation of the RNA (2 mg) is achieved when an equivalent amount (1 mg) on a residue (charge) basis of polylysine is added. Analysis for nitrogen and phosphorus showed the washed precipitate to have a lysyl:nucleotide residue ratio of 1. (The nitrogen and phosphorus values are represented in Figure 1 as closed and open circles and have been normalized to the ordinate.)

Fractional amounts of RNA are precipitated at polylysine:RNA ratios short of residue equivalence but above 1:2. In all cases, the precipitated complex showed by analysis the 1:1 stoichiometry of lysyl:nucleotide residue. At these polylysine:RNA ratios no free polylysine could be detected in the supernatant solution by ninhydrin or by a more sensitive red-cell flocculation test.

Soluble Polylysine–RNA Complexes. At still lower polylysine:RNA ratios, soluble complexes were formed. The existence of the soluble complex was established by exclusion chromatography with the use of Sephadex G-75. A 1:5 complex (lysyl:nucleotide residue) emerged at the excluded position and contained all the polylysine as well as the RNA, whereas polylysine alone was retarded and emerged near the salt boundary.

Formation of Nuclease-Resistant Polylysine–RNA Complexes. These soluble 1:5 polylysine–RNA complexes were then exposed to the action of ribonucleases. During the course of digestion, a white precipitate formed and remained throughout digestion. This precipitate contained both polylysine and RNA, and analysis for nitrogen and phosphorus showed the composition to be equivalent proportions of nucleotide and lysine as in the insoluble polylysine–RNA complexes formed by direct titration.

That the persistence of the precipitate was not due

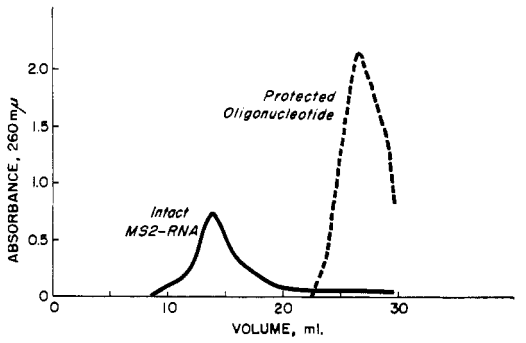

FIGURE 2: Sucrose density-gradient centrifugation. Performed in a Spinco SW-25 rotor in 0.01 M Tris·HCl, pH 7.3, 0.05 M NaCl, and 5–25% sucrose, at 0°, and 25,000 rpm for 20 hr. Thirty fractions of 1 ml were collected. Solid line: heated MS2-RNA. Dashed line: oligonucleotide fragment isolated after protection by polylysine "30" from digestion by micrococcal or *B. cereus* nuclease. See Table II.

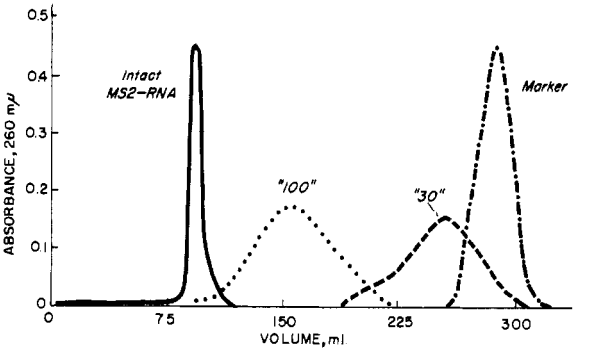

FIGURE 3: Composite of exclusion chromatography on Sephadex G-200. A 1.2 × 180 cm (internal diameter × height) column was equilibrated in a buffer consisting of 1 M NaCl and 0.01 M Tris·HCl, pH 8.0. The samples (0.2 mg) were applied in 1 ml of starting buffer and the column was developed at 4° at a constant flow rate of 7.5 ml/hr, two fractions per hour. Solid line: intact MS2-RNA. Dotted line: oligonucleotide fragment isolated after protection by polylysine "100" from digestion by *B. cereus* nuclease. Dashed line: similar fragment after protection by polylysine "30." Dashed dotted line: hexanucleotides (0.2–0.3 mg) derived from a partial T_1 ribonuclease digest of yeast RNA.

to inactivation of the nuclease was established by quantitative hydrolysis of freshly added RNA and by finding no further change in the absorbancy of the supernatant after the addition of fresh nuclease. However, the chain length of the polylysine used did affect the stability of the precipitate. Oligomers of lysine shorter than the heptamer afforded only transient protection since the precipitates which formed disappeared by the end of the digestion period.

The enzymatic action of micrococcal, *B. cereus*, pancreatic, or T_1 ribonucleases on the soluble 1:5 complex resulted in the formation of insoluble, nuclease-resistant polylysine–RNA complexes. These precipitated complexes all contained equal or similar amounts of RNA material, close to the theoretical value of 20% for the 1:5 complex. However, micrococcal and *B. cereus* nucleases, requiring Ca^{2+} for activity, could be inactivated by EDTA, thus precluding further nuclease action after dissociation of the nuclease-resistant complex. Pancreatic and T_1 ribonuclease, on the other hand, could not be easily removed or inactivated; hence most experiments were performed with micrococcal and *B. cereus* nucleases.

Protected RNA Fragment. At the end of the incubation period, the nuclease-resistant, polylysine–RNA precipitate was removed by centrifugation, washed, and dissolved in a solution containing EDTA. The solution, 7 M urea–0.10 M ammonium carbonate, pH 8.6–0.01 M EDTA, also served to dissociate the polylysine–RNA complex into its components which were separated as well as desalted on DEAE-cellulose. Exposure of the nuclease-resistant, polylysine–RNA precipitate to Pronase (but not to trypsin[2]) also brought about the solution of the complex by digestion of the polylysine to monomers and very short oligomers of lysine and permitted the recovery of a RNA fragment. The yield of protected RNA material so isolated ranged from 12 to 19 mg of RNA/10 mg of polylysine. Yeast, soluble, and heat-denatured MS2-RNA generally gave higher yields of protected RNA than tobacco mosaic virus (TMV) or unheated (native) MS2-RNA.

The yield of protected RNA could be doubled by a twofold increase in the ratio of polylysine:RNA (*i.e.*, a 1:2.5 complex). Higher proportions of polylysine led to the formation of a precipitate before nuclease addition.

The protected RNA fragments obtained from the *B. cereus* and micrococcal nuclease procedures were characterized in several ways. (1) The isolated fragments were shown to be reduced to mononucleotides and mono- and dinucleotides, respectively, on reincubation with these two enzymes. (2) That no RNA molecules were completely protected and that all RNA molecules were attacked by the nuclease was shown by sedimentation of the RNA fragment through a sucrose density gradient (Figure 2). None of the original RNA was found under conditions where less than 1% would have been detected. In a similar experiment, less than 3% of the original amino acid acceptor activity of s-RNA could be recovered from the isolated protected s-RNA fragment. This value is within the errors of the measurements. (3) Column chromatography on Sephadex G-200 revealed the absence of the original RNA (Figure 3) as well as size differences between the protected RNA segments prepared with polylysine "30" and polylysine "100."

Micrococcal and *B. cereus* nucleases were employed because of their metal requirements for activity and the nonspecific nature of their hydrolytic action.

TABLE I: Chain Length of Protected RNA Segment.

RNA	Polylysine Chain Length	Nuclease[a]	Chain Length Determination[b]	
			Total P/ Terminal P	Phosphatase, Then Alkali
Yeast RNA	—	—	>250	
	"30"	*B. cereus*	>100	>100[c]
Yeast s-RNA	—	—	~60	
	"30"	Micrococcal	28–30	
TMV-RNA	—	—	>1000	
	13	Micrococcal	16	15
MS2-RNA	—	—	>400	
	"30"	Micrococcal	28[d]	
	"30"	Micrococcal	38[e]	
	21–24	Micrococcal	28	
MS2-RNA (heated)	—	—	>400	
	"30"	*B. cereus*	>400	>400[c]
	"100"	*B. cereus*	>400	>400[c]
	"30"	Micrococcal	29–33	43
	"100"	Micrococcal	99	

[a] For digestion conditions see text and footnote to Table III. [b] Values listed as greater than (>) are minimal values and are derived from the smallest detectable amount of terminal phosphate (P) or ribonucleoside released by alkaline phosphatase or alkaline hydrolysis, respectively. [c] Values also obtained without prior alkaline phosphatase treatment. [d] Preincubation with polylysine and digestion with nuclease in 0.01 M Tris·HCl, pH 8.5. [e] Preincubation with polylysine and digestion with nuclease in 0.4 M Tris·HCl, pH 8.5.

Moreover, both enzymes pass through DEAE-cellulose by washing with 0.1 M NH_4HCO_3, pH 8.6 (which does not elute the RNA fragments). In contrast, the use of pancreatic or T_1 ribonuclease is complicated by difficulties encountered in their removal or complete inactivation during solubilization of the polylysine–RNA complexes. Therefore, because of the probability that traces of active nucleases were still present, protected fragments resulting from the action of these latter two enzymes could not be examined for chain length by alkaline phosphatase treatment or exclusion chromatography.

Chain Length of Protected RNA Fragment. When the protected fragments were prepared by the micrococcal nuclease procedure, chain length determinations by terminal *vs.* total phosphate showed a definite correlation between the chain length of polylysine used and that of the protected RNA obtained (Table I). This was independent of the RNA source. Essentially the same chain length ratios (close to unity) were obtained by nucleoside determinations after terminal phosphate release.

Protected segments prepared by the *B. cereus* ribonuclease treatment, on the other hand, showed neither a phosphomonoesterase-labile phosphate nor a terminal ribonucleoside, precluding chain length determinations by the measurements of these terminal groups. Phosphodiester cleavage by *B. cereus* nuclease proceeds *via* a 2′,3′-cyclic phosphate intermediate (G. W. Rushizky, unpublished observations). In the case of the protected fragments, hydrolysis by *B. cereus* nuclease appears to stop at the cyclic phosphate stage, which is known to be resistant to dephosphorylation by alkaline phosphatase (Heppel *et al.*, 1962). However, sedimentation through sucrose density gradients (Figure 2) and exclusion chromatography with Sephadex G-200 established that RNA fragments prepared by either the *B. cereus* (Figure 3) or micrococcal nuclease procedures were of similar size, *i.e.*, emerged at the same effluent volumes.

Nucleotide Composition of Protected Fragment. Nucleotide determinations revealed no or only small differences in composition between the original RNA and the protected RNA fragment in the case of yeast RNA, s-RNA,[3] or heated MS2-RNA (Table II). By contrast, complexes formed with "native" (*i.e.*, unheated) MS2-RNA as well as TMV-RNA yielded nuclease-resistant fractions which differed significantly in base ratio from that of the original RNA (Tables II and III). Thus, the proportions of Gp and Cp were higher and that of Ap and Up lower than the corresponding values for the original RNA. However, expo-

[3] Yeast s-RNA, approximately 60 nucleotides in length, has a high Gp + Cp content (Table II). Thus, any specific protection by polylysine "30," involving about one-half of the s-RNA molecule, would not be reflected in the nucleotide composition of the protected fragment.

TABLE II: Nucleotide Composition of Protected RNA.[a]

RNA	Polylysine Chain Length	Nuclease	Nucleotide Composition, % Cp	Ap	Gp	Up	(Gp + Cp)/(Ap + Up)
Yeast RNA	—	—	21	25	29	25	1.0
	"30"	*B. cereus*	19	24	31	25	1.0
	"30"	Micrococcal	22	23	30	24	1.1
Yeast s-RNA	—	—	29	20	31	20	1.5
	"30"	Micrococcal	29	18	32	21	1.6
	"30"	Micrococcal	29	17	32	20	1.6
TMV-RNA	—	—	19	28	25	29	0.77
	"30"	*B. cereus*	28	19	33	20	1.6
	25–30	Micrococcal	33	14	42	13	2.8
MS2-RNA	—	—	26	23	27	24	1.1
	"30"	*B. cereus*	33	14	37	16	2.3
	"30"	Micrococcal	33	13	42	12	3.0
	25–30	Micrococcal	37	11	39	13	3.2
MS2-RNA (heated)	—	—	26	23	27	24	1.1
	"30"	*B. cereus*	26	21	29	24	1.2
	"100"	*B. cereus*	27	20	29	24	1.3
	"30"	Micrococcal	26	21	29	24	1.2
	"100"	Micrococcal	26	20	29	25	1.2

[a] See text for details.

sure of this "native" MS2-RNA to heat (Table III) resulted in a decrease of the high Gp and Cp content of the protected RNA fragment but increased the yield.

The yield of protected fragment was also inversely related to the salt concentration employed (Table IV), with increasing salt decreasing the amount of protected fragment obtained. High Gp and Cp composition was maintained with "native" MS2-RNA even in the presence of 0.4 M Tris–HCl although the yield was markedly reduced.

Because of the uncertainty of inactivation of pancreatic and T_1 ribonuclease, nuclease-resistant, polylysine–RNA precipitates prepared with these enzymes were taken up directly in 1 N KOH. Nucleotide composition analyses revealed that the RNA fragments were very similar to "cores" (Zamenhof and Chargaff, 1949). The presence of 0.001 M $CaCl_2$ or $MgCl_2$ did not alter the nucleotide distribution. In the case of MS2-RNA, the nucleotide compositions found in the protected fragments prepared with pancreatic and T_1 ribonuclease digestion were 12:27:50:11 and 27:23:20:31 for Cp:Ap:Gp:Up, respectively, while actual "core" values obtained in our laboratory were 10:34:42:14 and 27:23:19:31, respectively. Moreover, precipitates of similar composition were obtained when polylysine was added to unprotected MS2-RNA *after* exhaustive digestion by pancreatic and T_1 ribonuclease.

Discussion

One of the objectives of this investigation was to develop procedures for the isolation of nucleic acid fragments with chain lengths of 10 to 100 nucleotide residues for use in sequence determinations of high molecular weight RNA. The results show that such fragments can be obtained by protection with polylysine. Selection of the chain length of the protecting lysine oligomer (larger than the heptamer) determines the chain-length range of oligonucleotides obtained. The yield of RNA fragments prepared by nuclease digestion of soluble polylysine–RNA complexes (1:5) can only be 20%, a value that has been achieved in our experiments. This value represents a considerably greater yield of oligomers (larger than the decamer) than that obtained (less than 1%) from unprotected high molecular weight RNA by complete digestion with specific nucleases. Furthermore, RNA segments obtained in this manner contain nuclease-sensitive sequences which overlap those obtained by conventional enzymatic techniques.

B. cereus and micrococcal[4] nucleases were found to

[4] While micrococcal nuclease exhibits preferential cleavage at Ap and Up linkages (Roberts *et al.*, 1962), complete digestion by this enzyme is relatively nonspecific, *i.e.*, only mono- and dinucleotides are found.

TABLE III: Effect of Heat on Yield and Nucleotide Composition of Protected RNA.[a]

Exposure of MS2-RNA to 100° (min)	Yield of Protected RNA[b] (%)	Nucleotide Composition, % Cp	Ap	Gp	Up	(Gp + Cp)/(Ap + Up)
0	63	33	14	37	16	2.3
10	91	28	19	30	23	1.4
30	102	27	22	27	24	1.2

[a] Polylysine "30":MS2-RNA ratio, 1:5. MS2-RNA was heated at 1.0 mg/ml in 0.001 M $CaCl_2$ and 0.01 M Tris·HCl, pH 7.5, as indicated and then chilled rapidly to 0°. Polylysine "30" was added and digestion was performed with *B. cereus* ribonuclease at 40° in 0.01 M Tris·HCl, pH 7.5. The precipitate was isolated and the nucleotide composition of the RNA segment determined as described in the text. [b] At the 1:5 lysyl:nucleotide ratio, 100% yield of RNA in the precipitated complex would be 20% of the original RNA added.

TABLE IV: Effect of Salt Concentration on Yield and Nucleotide Composition of Protected RNA.[a]

Tris·HCl, pH 8.5 (M)	Yield of Protected RNA[b] (%)	Nucleotide Composition, % Cp	Ap	Gp	Up	(Gp + Cp)/(Ap + Up)
0.01	85	32	14	38	17	2.3
0.10	70	33	14	38	16	2.4
0.40	46	33	14	37	16	2.3

[a] Conditions as in Table III except for the variation in salt concentration, as tabulated, during the formation of the soluble polylysine–RNA complex and during nuclease digestion. [b] At the 1:5 lysyl:nucleotide ratio, 100% yield of RNA in the precipitated complex would be 20% of the original RNA added.

be particularly suitable because of the facility by which they could be inactivated and because mono- and dinucleotides were the end products of their hydrolytic action. No "cores" were produced. Pancreatic and T_1 ribonuclease, on the other hand, were difficult to inactivate and produced large amounts of "core" material.

Another disadvantage to the use of these latter two specific nucleases derives from the absolute specificity of their hydrolytic action. Thus, enzymatic hydrolysis of the soluble polylysine–RNA complex would not stop at the complexed polylysine segment, but at the nearest unprotected pyrimidine or guanylic acid residue. The protruding nucleotide sequences might then render the resulting nuclease-resistant, polylysine–RNA sequence more soluble and would certainly modify the chain length relationships of the protecting polylysine and the protected RNA segment.

The major findings reported in this paper are the protection of RNA segments by polylysine against nuclease action, the equivalent stoichiometry and the apparent collinearity of the insoluble complex, and, under appropriate conditions, the specificity of this protection. Specificity of protection, producing fragments with a high Gp + Cp content, appears to be related to the ordered structure of the nucleic acid since it is markedly reduced by thermal denaturation of the RNA prior to polylysine addition. These results impose certain constraints on the acceptable models for polylysine–RNA interaction and stimulate speculation on the implications of this interaction and the protective effect it affords.

The near-unity ratio of lysine:phosphate in the protected or insoluble complex is consistent with the correlation between charge neutralization and insolubility (Spitnik *et al.*, 1955), and with the protection of DNA against nuclease action by poly-4-vinylpyridine charged to various degrees by quaternization (Bach and Miller, 1966). Our data are also consistent with conductometric studies (Felsenfeld and Huang, 1959) which have shown that polylysine displaces an equivalent amount of divalent cation (Mn^{2+}) from polynucleotides. The finding (Shulman *et al.*, 1965) from nuclear magnetic resonance measurements that Mn^{2+} binds to the polynucleotide phosphate groups is pertinent in this connection. These observations provide support to the concept that protection of RNA against nuclease action is afforded by the electrostatic binding of the positively charged ϵ-amino groups of polylysine to the RNA phosphate groups.

The unit ratio of average chain length of protected RNA segment to that of the protecting polylysine greatly restricts the spatial relationships by which the lysyl:nucleotide residue ratio of 1 can be achieved. It is unlikely that random cross-linking between RNA chains by polylysine is the principal mechanism since the average chain length of a protected RNA segment would then be the ratio of total to uncomplexed phosphates, a ratio which would be relatively insensitive to polylysine chain length. Thus a model involving a general collinearity between the RNA and the complexing polylysine seems obligatory.

Such a model consistent with our data could have the polylysine interacting with single-stranded or double-stranded regions of the RNA molecule. The former would involve an extended collinear complex of RNA and polylysine in which one was coiled around the other so that corresponding phosphates and ϵ-amino groups were close to one another. Such coiling

might require energetically unfavorable conformations of the RNA and polylysine. It would be restricted by the maximum possible residue translation in the direction of the polypetide chain of 3.6 A (Schellman and Schellman, 1964) and the maximum possible interphosphate distance of about 9.8 A, the sum of the covalent distances between all atoms connecting two phosphorus atoms (Coulson, 1959). Furthermore, the single-stranded model does not predict the observed dependence of the protection on higher order structure since it would not be affected by thermal denaturation.

We consider interaction with double-stranded regions of the RNA to be more likely because it does predict the secondary structure dependence and base composition complementarity of the protected fragment which was actually observed. In fact, this model is somewhat similar to one constructed by Feughelman *et al.* (1955) for nucleoprotamine from X-ray studies and described later by Tsuboi *et al.* (1966). In that model, the polybase lies in an extended form along a groove of the nucleic acid helix with alternate basic residues binding to each phosphate of the two polynucleotide strands on either side of the groove. However, their model predicts a 1:2 ratio of protected polynucleotide segment length: polylysine length, a ratio inconsistent with our chain length data for RNA–polylysine complexes.

A more satisfactory modification of this model which is consistent with our chain length data and residue stoichiometry considers the polynucleotide strands to be two arms of a single "hairpin" coil in which the connecting loop is close enough to the polylysine to be itself protected. The few extra bases in this loop (a minimum of three according to Fresco, 1963) would not significantly affect these considerations. Moreover, the actual average protected RNA chain length values that are found were slightly greater than those of the polylysine used for protection.

Protection specificity would then be a consequence of polylysine complexes formed with *preformed* double-stranded, base-paired portions of the RNA molecule. Such specificity would disappear on thermal denaturation. (Recent studies by Bishop (1966) with coliphage RNA conclude that conformational changes rather than molecular degradation result from irreversible thermal denaturation.) Nonspecific protection, on the other hand, unaffected by heat, would be a result of complexes formed with *potential* double-stranded, but not necessarily base-paired, portions of the molecule. The high Gp + Cp content of protected fragments observed under conditions of specific protection could be related to the secondary structure of the RNA which the local base composition dictates (helical regions having a higher Gp + Cp content than the molecule as a whole), or it may be a consequence of relatively small nonelectrostatic interactions which direct the polylysine toward those helical regions which have a high Gp + Cp content. A number of reports have appeared with evidence for regions of secondary structure in native RNA, such as for MS2-RNA (Strauss and Sinsheimer, 1963), for s-RNA (Felsenfeld and Cantoni, 1964), and for ribosomal RNA (Cox, 1966).

It should be pointed out that the *protection* specificity we observe may not necessarily reflect *binding* specificity. This discrepancy could exist if the polylysine bound reversibly to the RNA in a random manner but maintained the ability to slide along the RNA. A higher susceptibility to nuclease action of coil regions as opposed to helical regions (reported for micrococcal nuclease by Dirksen and Dekker, 1960), or for Ap and Up linkages (Roberts *et al.*, 1962), would result in the preferential hydrolysis of these regions and the ultimate binding of the polylysine to the helical and high Gp + Cp regions.

Protection specificity need also not be related to the aggregation tendency of polynucleotide regions complexed with polycations. Investigation of this effect is being carried out by Leng and Felsenfeld (1966) in a system much different from ours, one in which polylysine or polyarginine selectively precipitate out certain fractions of DNA at very high salt concentrations (0.5–1 M) and higher lysyl to nucleotide residue ratios. Their experiments show a preferential precipitation by polylysine of DNA rich in A–T pairs and a preferential precipitation by polyarginine of polynucleotides rich in G–C pairs, both in 1 M sodium chloride, with the polylysine selectivity reversed if tetramethylammonium chloride is used as the salt.

Nuclease resistance induced by the interaction of RNA with polyamines (or proteins) may play an important role in controlling the stability of messenger ribonucleic acid (m-RNA) as suggested also by Armentrout *et al.* (1966) and could, at least in part, be responsible for the relative stability of ribosomes in biological systems. The stability of m-RNA and s-RNA bound to ribosomes (Takanami and Zubay, 1964; Takanami *et al.*, 1965; Cannon *et al.*, 1963) may be a consequence of this protection and thereby be related to the kinetics of protein synthesis. Transport of nucleic acids through membranes has been facilitated by partial charge neutralization of RNA by polyamines (Amos, 1961). Masking of biological function of RNA by basic proteins has been proposed for some time primarily on the basis of circumstantial evidence. More recently, Monroy *et al.* (1965) have shown that trypsin treatment of ribosomes from unfertilized sea urchin eggs was followed by increased protein synthesis in response to exogenous messenger as well as increased endogenous incorporating activity. Skalka *et al.* (1966) have reported polycation inhibition of RNA synthesis which was proportional to the adenine plus thymine content of the DNA primer. The product formed in the presence of histones was both smaller and of different base ratios and sequence than that formed in the absence of inhibitor.

The protection specificity we observe, even if it does reflect binding specificity, does not appear to be high enough to be of biological consequence. However, since relatively long chain polylysines were used in our protection experiments, the specificity observable by the methods used was, perforce, limited. In biological systems, proteins with separated short runs of basic

amino acids might possess a great deal of information for nucleic acid sequence specificity, particularly when aided by the nonelectrostatic interactions possible between the amino acid side chains of such proteins and the nucleic acid. This latter effect might provide the additional fine adjustment needed for biological specificity, with the electrostatic interaction serving largely to provide sufficient binding strength.

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