Effects of Poly-L-Lysine on Infectious Viral Nucleic Acid

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Infectious ribonucleic acids (IRNA) of Venezuelan equine encephalitis and Eastern equine encephalitis viruses were observed to form noninfectious complexes with a basic polyamino acid, poly-L-lysine. Original infectivity was recovered from the complexes by digestion of the polylysine with Pronase, and partial recovery was effected by treatment with sodium dodecyl sulfate. Infectivity could not be recovered from the complexes containing polylysine of 100,000 molecular weight by changes in ionic strength, pH, or by treatment with phenol, deoxycholate, or digitonin. Masking of infectivity by polylysine was demonstrated *in vivo* as well as by plaque assay in tissue culture. Poly-L-lysine preparations of high molecular weight (44,000 to 100,000) were more effective than low molecular weight (3,000) materials in masking infectivity of IRNA. When complexes, in which infectivity had been masked by low molecular weight polylysine, were suspended in 1 M NaCl, some infectivity was recovered. Complexes of polylysine-IRNA differed from control IRNA alone in (i) resistance to inactivation by ribonuclease, (ii) sedimentation patterns in sucrose gradient centrifugation, and (iii) stability of recoverable infectivity during different physical treatments.

Proteins, especially histones, when coupled with nucleic acids have been shown to affect enzymatic processes, to stabilize the functional structure of double-stranded nucleic acids, and to protect the latter against thermal or radiational damage (4). Nucleic acids or polynucleotides complexed with proteins have been observed to act as haptens in immunological processes (10). Tsuboi and others (3, 6, 13) have described interaction between double-stranded nucleic acids and a synthetic basic polyamino acid, poly-L-lysine, and the resulting changes in physical properties (e.g., melting temperature profiles, X-ray diffractions, and electrophoretic mobility).

In 1967 Norrell and Costlow (9) presented evidence that methylated bovine serum albumin formed, reversibly, noninfectious complexes with the infectious ribonucleic acids (IRNA) from Venezuelan equine encephalitis (VEE) virus. We found that poly-L-lysine of 100,000 molecular weight was more efficient than methylated bovine serum albumin in "masking" infectivity of IRNA from VEE or Eastern equine encephalitis (EEE) viruses. We have examined the nature of polylysine-IRNA and the effect of a number of factors, particularly in respect to the biological activity of IRNA in the presence of the polyamino acid.

MATERIALS AND METHODS

Viruses. EEE virus, CDC strain SC7, was propagated in monolayer primary cultures of chick embryo fibroblast (CEF) cells, and VEE virus strain described by Hardy (2) was grown in monolayer cultures of CEF or L cells. Supernatant growth medium was harvested from the infected cultures and centrifuged for 15 min at $500 \times g$ to remove cell debris. Virus was then pelleted by centrifugation for 2 hr at $67,000 \times g$ and suspended to one-tenth of the original volume in 0.02 M phosphate buffer, pH 7.4, containing 0.001 M disodium ethylenediaminetetraacetate (EDTA).

Extraction of IRNA. IRNA was extracted from the concentrated virus suspension by treatment with phenol and sodium dodecyl sulfate (SDS). First, the virus suspension was treated, in the presence of 0.17 M SDS, with 2 volumes of water-saturated phenol at 50 C. The aqueous phase was separated by centrifugation and reextracted at 22 C with 1 volume of water-saturated phenol. Potassium acetate was added to the separated aqueous phase of the second extraction to a final concentration of 2%, and the IRNA was precipitated by the addition of 3 volumes of cold ethanol. After 1 hr or longer at -15°C the precipitate was collected by centrifugation and resuspended in the buffered phosphate containing EDTA. Preparations were stored at -60°C in volumes appropriate for use in individual experiments. The infectivity of

the IRNA preparations was 0.02 to 0.05% that of the virus from which they were extracted. Specific infectivity of individual IRNA preparations was from $10^{4.6}$ to $10^{5.7}$ plaque-forming units (PFU)/ μ g of RNA.

Assay of RNA. Infectivity of IRNA, as indicated by plaque assay, was determined as previously described (1) with hypertonic NaCl on monolayer cultures of CEF cells. In some experiments as noted in the text, 0.15 M NaCl, buffered with tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride at pH 8.2, was substituted for 1 M NaCl as diluent for the IRNA or polylysine-IRNA. For assays of infectivity in vivo, suckling Swiss albino mice or 3- to 4-day-old White Leghorn chicks were inoculated by the intracerebral route with IRNA or polylysine-IRNA suspended in buffered 0.15 M NaCl (0.02 ml per mouse and 0.1 ml per chick, respectively). RNA concentration was estimated from the absorbance of concentrated preparations at 260 nm by using the molar extinction coefficient for yeast RNA (35.9×10^3) determined by Schwarz BioResearch, Inc., Orangeburg, N.Y.

Polylysine-IRNA preparations. Suspensions of polylysine or IRNA were prepared at twice the desired final concentrations in 0.02 M PO_4 buffer, pH 7.4, or in Tris-buffered 0.15 M NaCl. Immediately before each experiment was performed, the polylysine solution was added slowly, with agitation, to the suspended IRNA at 22 C. The preparations were incubated for 5 min at 22 C and then held at 2 to 4 C in a water-ice bath until treated or assayed for the experiments. Ratios for polylysine:RNA are given as w/w. The concentration of RNA used for preparation of the complexes was adjusted for the requirements of individual experiments, and unless otherwise stated the final concentration of RNA in the complexes was $< 5 \mu\text{g/ml}$.

Materials. Poly-L-lysine-hydrochloride preparations of 100,000 molecular weight were obtained from Mann Fine Chemicals, Inc., New York, N.Y., and poly-L-lysine-HBr of lower molecular weight was from Miles Laboratories, Inc., Elkhart, Ind. Crystalline ribonuclease (EC 2.7.7.16) from bovine pancreas, specific activity approximately 2,500 units per mg, was purchased from Worthington Biochemical Corp., Freehold, N.J. Pronase (*Streptomyces griseus protease*), B grade, specific activity of 45 units per mg, was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Effect of polylysine on infectivity of IRNA. Suspensions of polylysine-IRNA were prepared in 0.02 M PO_4 buffer, pH 7.4, to contain the desired ratios of high molecular weight (100,000) polylysine to RNA. These suspensions were then diluted in duplicate, in 0.15 M NaCl and 1 M NaCl diluents, for plaque assay. Data (Table 1) from a typical experiment with VEE IRNA indicated that polylysine, when present in twice the concentration of RNA, significantly masked infectivity of IRNA. This loss of infectivity was observed when the polylysine-IRNA was sus-

TABLE 1. *Effect of high molecular weight polylysine on infectivity of infectious ribonucleic acids of Venezuelan equine encephalitis virus*

Ratio of polylysine to RNA (w/w)	Infectivity ($\log_{10}$ PFU/ml) ^a	
	In 0.15 M NaCl	In 1 M NaCl
0:1	5.6	6.3
1:1	5.5	5.9
2:1	3.2	<2.7
5:1	<2.7	<2.7

^a PFU, plaque-forming units.

pended in either 0.15 M or 1 M NaCl. From these results it was not apparent whether the IRNA formed complexes with polylysine that were not dissociated in 1 M NaCl or whether it was denatured in the presence of polylysine.

Sober et al. (12) reported that Pronase treatment of complexes of polylysine and oligonucleotides of yeast RNA dissociated the two components without altering the oligonucleotide length. We examined whether Pronase digestion of high molecular weight polylysine-IRNA would effect recovery of the intact infectious unit of IRNA. Suspensions of polylysine-IRNA were prepared with appropriate ratios of polylysine:RNA. Portions of the mixtures and of IRNA alone were incubated with Pronase (1 unit/ml) at 37 C and pH 8.2. Samples were removed and assayed at once in 0.15 M NaCl diluent before Pronase treatment and at intervals during the treatment. Significant recovery of original infectivity was observed in a typical experiment with VEE IRNA (Fig. 1) when polylysine-IRNA preparations containing 0.5 to 2.5 times as much polylysine as RNA were treated with 1 unit of Pronase per ml. In 15 min at 37 C complete recovery of the original infectivity of the IRNA preparation was obtained from the complex containing 0.5 μ g of polylysine per ml. In 30 min the same concentration of Pronase in the presence of higher concentrations of polylysine effected significant though incomplete recovery of original infectivity of the IRNA. In some experiments, concentrations of Pronase up to 12.5 units/ml and incubation up to 45 min were employed successfully to obtain more efficient recovery of infectivity from preparations containing up to 4 μ g of polylysine per ml. However, because Pronase concentrations above 0.5 unit/ml caused damage to CEF cultures used in the assays, limitations were imposed on the Pronase concentration feasible in individual experiments, especially where IRNA preparations of lower specific infectivity were used. In some cases,

FIG. 1. Effect of Pronase treatment on infectivity of polylysine-infectious ribonucleic acids (IRNA). Complexes of polylysine and VEE IRNA were incubated for appropriate times with Pronase and then assayed for infectivity. (Infectivity of IRNA alone was the same before and after 30 min of Pronase treatment.) Concentration of polylysine and ratio (w/w) of polylysine:RNA: (■), 0.5 µg/ml and 0.5:1; (▲), 1.0 µg/ml and 1:1; and (●), 2.5 µg/ml and 2.5:1.

therefore, only partial recovery of the original infectivity was possible. Pronase treatment did not affect the infectivity of control IRNA alone, but some preparations of IRNA were not completely stable at 37 C.

Studies of the reversible infectivity loss of IRNA in the presence of polylysine (100,000 molecular weight) were performed with EEE IRNA in 3- and 4-day-old White Leghorn chicks and with VEE IRNA in 1- to 2-day-old Swiss albino mice. To verify specific deaths, virus was isolated from representative brain specimens and identified by plaque neutralization tests using specific hyperimmune sera. Data from a representative experiment in chicks are shown in Table 2. Doses of IRNA in PFU were determined for the inocula before the addition of the polyamino acid; plaque assays for suspensions of polylysine-IRNA at all of the concentrations used were negative. Polylysine, at 2.5 times the concentration of the RNA, significantly reduced the infectivity of EEE IRNA in baby chicks. Similar results were observed in experiments with polylysine-VEE IRNA in suckling mice. When polylysine-IRNA preparations were treated with Pronase before inoculation, mortality was similar to that obtained with IRNA alone.

Effect of ribonuclease on polylysine-IRNA. Preparations of IRNA in the presence and absence of polylysine (100,000 molecular weight) were treated with ribonuclease (0.0025 unit/ml) for 5 min at 37 C, diluted, and then treated with Pronase (1 unit/ml) for 30 min at 37 C. Data shown in Table 3 from an experiment with VEE

TABLE 2. Effect of polylysine on infectivity of infectious ribonucleic acids (IRNA) of equine encephalitis virus in four-day-old chicks

Dose of IRNA (PFU) ^a	Ratio of polylysine to RNA (w/w)	Mortality (%)
3,500	0:1	100
350	0:1	75
35	0:1	0
3,500 ^b	2.5:1	33
350 ^b	2.5:1	0
35 ^b	2.5:1	0

^a Plaque-forming units. Dosage given intracerebrally.

^b Represent PFU of IRNA before addition of polylysine. In presence of polylysine, no infectivity was demonstrated in these inocula by plaque assay.

TABLE 3. Effect of pancreatic ribonuclease on polylysine-infectious ribonucleic acids (IRNA)

Ratio of polylysine to RNA (w/w)	Treatment at 37 C		Titer of IRNA (log ₁₀ PFU ^b /ml)
	Ribonuclease at 0.0025 unit/ml (5 min ^a)	Pronase at 1.0 unit/ml (30 min)	
0:1	—	+	6.0
0:1	+	+	3.8
2:1	—	+	5.0
2:1	+	+	5.0

^a Immediately after treatment with ribonuclease, samples were diluted 20-fold (with addition of Pronase) to stop ribonuclease activity.

^b Plaque-forming units.

IRNA indicated that there was no significant difference in recoverable infectivity between ribonuclease-treated and control polylysine-IRNA, but IRNA alone was 99% inactivated by the nuclease treatment. For this study the polylysine:RNA ratio was 2:1. The effects of higher concentrations of ribonuclease have not been examined, as the dilution required to stop the nuclease activity during Pronase treatment would dilute the IRNA beyond the end point for infectivity.

Sucrose gradient centrifugation of polylysine-IRNA. For sucrose gradient centrifugation studies, two equal portions of an IRNA preparation were used. To one, polylysine (100,000 molecular weight) was added to give the desired polylysine:RNA ratio, and the second portion was diluted with phosphate buffer to the same concentration of RNA contained in the polylysine-IRNA preparation. The prepared samples

were centrifuged in separate sucrose gradients, and collected fractions were assayed for infectivity. Fractions from the polylysine-IRNA gradient were assayed before and after Pronase treatment. Data represented in Fig. 2 are from an experiment with VEE IRNA. Infectivity of the fractions is expressed as per cent of the total infectivity recovered from each centrifuged sample. A significant portion of the IRNA in the polylysine-IRNA preparation sedimented more rapidly than IRNA that was centrifuged alone. Not only was a higher percentage of infectivity observed in the lower portion of the gradient containing polylysine-IRNA, but the absolute infectivity recovered from the three bottom fractions was 100 times that found in the same fractions of the gradient containing IRNA alone, i.e., $10^{5.1}$ and $10^{3.0}$ PFU, respectively. Furthermore, when the inner surface of the bottom of the tubes was washed with 0.5 ml of buffer, an additional $10^{5.5}$ PFU of infectivity was recovered from the gradient that contained polylysine-IRNA compared with $10^{3.6}$ PFU from the tube that contained the control IRNA. Polylysine alone, centrifuged under the same conditions, remained in the two top fractions of the gradient.

Effect of molecular weight of polylysine on masking of infectivity. Four preparations of poly-L-lysine of molecular weights 3,000 to 100,000 were compared for their effectiveness in masking the infectivity of IRNA. All preparations were used at the same concentration. In a study with EEE IRNA (Table 4) polylysine and RNA were used in a ratio of 4:1, which resulted in >99% masking of infectivity when polylysine of 100,000 molecular weight was used. (With this IRNA preparation a 2:1 ratio of the high molecular weight polyamino acid did not significantly affect infectivity.) Each of the polylysine preparations in appropriate concentration was added to a separate sample of the IRNA preparation. The mixtures were assayed in 0.15 M NaCl before and after Pronase treatment. The data indicated that preparations of 44,000 to 100,000 molecular weight were equally effective at the concentration employed. The low molecular weight (3,000) material did not significantly mask infectivity. Also, infectivity of IRNA in polylysine-IRNA prepared with the low molecular weight (3,000) polyamino acid was not protected against inactivation by ribonuclease.

In an additional study, the low molecular weight polylysine was compared with the high molecular weight material at several concentrations. Data from a typical experiment with VEE IRNA are shown in Table 5. The polylysine-IRNA preparations were diluted in duplicate,

FIG. 2. Sucrose gradient centrifugation of polylysine-infectious ribonucleic acids (IRNA). Complexed VEE IRNA (2.2 μ g of polylysine and 12.5 μ g of RNA in 1.1 ml, original infectivity of IRNA 80% masked) and VEE IRNA alone (12.5 μ g of RNA, $10^{8.8}$ plaque-forming units in 1.1 ml) were layered onto separate gradients of 10 to 28% sucrose in 0.02 M PO_4 buffer, pH 7.4, containing 0.001 M EDTA. They were centrifuged for 3.5 hr at $57,000 \times g$ in an SW 39 rotor of a Spinco model L centrifuge. Six-drop fractions were collected from the bottom of the tubes and assayed for infectivity; fractions from the complexed sample were assayed before and after Pronase treatment. Infectivity is expressed as per cent of total infectivity recovered from each centrifuged sample. (●), Fractions of uncomplexed RNA; (▲), untreated fractions of polylysine-IRNA; and (△), Pronase-treated fractions of polylysine-IRNA.

in 0.15 M and in 1 M NaCl, for assay. In isotonic diluent when the low molecular weight (3,000) polyamino acid was employed, a 10:1 polylysine:RNA ratio was required to effect masking similar to that obtained with a 2:1 ratio of the high molecular weight preparation. Some recovery of infectivity was obtained when IRNA

TABLE 4. *Effect of molecular weight of polylysine on reversible masking of infectivity of infectious ribonucleic acid (IRNA)*

Sample	Molecular weight of polylysine	Titer ($\log_{10}$ PFU/ml) ^a	
		Un-treated	Pronase-treated
IRNA alone.....		5.8	5.5
Polylysine-IRNA.....	3,000	5.5	5.1
Polylysine-IRNA.....	44,000	<2.7	4.8
Polylysine-IRNA.....	75,000	<2.7	5.0
Polylysine-IRNA.....	100,000	<2.7	4.9

^a PFU, plaque-forming units.TABLE 5. *Effect of low and high molecular weight polylysine on infectivity of infectious ribonucleic acids of Venezuelan equine encephalitis virus*

Ratio of polylysine to RNA (w/w)	Molecular weight of polylysine	Infectivity ($\log_{10}$ PFU/ml)	
		In 0.15 M NaCl	In 1 M NaCl
0:1		5.5	6.1
2:1	100,000	3.2	<2.7
5:1	100,000	<2.7	<2.7
10:1	100,000	<2.7	<2.7
2:1	3,000	5.1	6.1
5:1	3,000	4.4	5.5
10:1	3,000	3.0	4.7

masked with the low molecular polylysine was brought to 1 M NaCl concentration. Infectivity of all samples after treatment with Pronase was similar to that of Pronase-treated control IRNA alone.

When similar studies were performed with both EEE and VEE IRNA and L-lysine, no effect on infectivity was observed in the presence of lysine concentrations up to 1,000 times that of the RNA.

Effect of pH of suspending medium on masking effect of polylysine. Polylysine (molecular weight 100,000) and VEE IRNA were suspended separately in Michaelis buffers of pH range 6.0 to 9.0. The buffers were prepared in steps of approximately 0.5 pH units. Polylysine, at each pH, was added to IRNA suspended in buffers of like pH value in a polylysine:RNA ratio of 2:1. Each polylysine-IRNA sample was then diluted in the corresponding Michaelis buffer for plaque assay. The 1 M NaCl used for the preinoculation treatment of the CEF cultures used in the assays was adjusted with Tris-hydrochloride to correspond with the pH of the individual samples. For each

pH value, samples of control IRNA alone were assayed in the same manner. At each pH value over the range 6.0 to 9.0, the infectivity of IRNA in the presence of polylysine was found to be <1% compared with the infectivity of control IRNA.

Effect of various conditions and treatments on recovery of infectivity from polylysine-IRNA. Two different preparations of VEE IRNA and polylysine of 100,000 molecular weight were used to study the effect of storage at 4 and -60 C on the infectivity recoverable by Pronase treatment of polylysine-IRNA. Polylysine:RNA ratio of the samples was approximately 3:1, and all samples were suspended in 0.02 M PO_4 buffer, pH 7.3, containing 0.0005 M EDTA. One-tenth milliliter portions of each sample, with or without polylysine, were stored in small polyethylene tubes with snap caps. At intervals representative samples were removed from storage and treated at 37 C for 45 min with 5 units of Pronase per ml and then assayed for infectivity. To overcome possible absorption of the complex to the tubes, enzyme treatment was performed directly in the tubes in which the samples had been stored. Data obtained with one of these preparations are shown in Table 6. At -60 C there was no loss of infectivity in control samples of IRNA alone over a period of 12 weeks, but after the same period of time only one-third of the original infectivity could be recovered from the polylysine-IRNA. At 4 C, the control IRNA lost about one-third of its original infectivity by 2

TABLE 6. *Effect of polylysine on recoverable infectivity of stored infectious ribonucleic acids (IRNA) of Venezuelan equine encephalitis virus*

Storage conditions		Titer of IRNA ($\log_{10}$ PFU/ml) ^a	
Temp (C)	Time (weeks)	RNA alone	RNA + polylysine ^b
-60	0	5.5	5.5
-60	2	5.5	5.2
-60	5	5.6	5.3
-60	12	5.5	4.9
4	0	5.5	5.5
4	2	5.3	4.3
4	5	5.3	3.8
4	10	5.2	3.0

^a Infectivity was assayed after treatment with 5 units of Pronase per ml at 37 C for 45 min. PFU, plaque-forming units.

^b Polylysine: RNA ratio was approximately 3:1 (w/w).

weeks and then remained stable throughout the remainder of the 10-week test period. However, less than 10% of the original infectivity was recovered from polylysine-IRNA after 2 weeks and less than 0.5% after 10 weeks at 4 C. Results with the second IRNA preparation were qualitatively similar although the titer of the control IRNA in that study dropped to about 25% of the original value in 10 weeks at 4 C. Again, however, the recoverable infectivity of the complexed IRNA declined much more rapidly than that of the control and less than 0.5% could be recovered after 10 weeks at 4 C.

Portions of one of the complexed preparations from the preceding experiment were heated to 55, 75, and 100 C to determine whether heating might loosen bonding in the stored complex and permit recovery of the original infectivity. Samples of complexed IRNA and parallel samples of IRNA alone, after storage at -60 C for 36 months, were incubated for 2 min at the appropriate temperatures in 0.02 M phosphate buffer containing 0.001 M EDTA, pH 7.4. Immediately after heating, the samples were treated with 1 unit of Pronase per ml at 37 C for 45 min. Final concentration of polylysine during the enzyme treatment was 0.2 µg/ml. Infectivity of samples was determined by plaque assay. Unheated samples were assayed before and after Pronase treatment. Data from a typical experiment (Table 7) indicated that much less infectivity was recovered by Pronase digestion of the heated complex than from the unheated complexed preparation. IRNA alone, on the other hand, was stable at 55 and 75 C and relatively stable at 100 C.

In related studies portions of the heated samples from the preceding experiment were added, without prior cooling, to a cold suspension of chick embryo tissue RNA to give a final ratio of polylysine: RNA of 1:2,500. If dissociation of polylysine and intact IRNA had occurred on heating, this excess of RNA should have eliminated the masking effect of polylysine on the IRNA. However, no infectivity was recovered from complexed samples that were heated and treated with excess RNA though, for uncomplexed IRNA treated in parallel, all of the original infectivity was recovered after treatment at 55 or 75 C, and significant infectivity remained after incubation at 100 C.

As it was necessary in some experiments to incubate or hold prepared complexes, the effect of time on the Pronase-recoverable infectivity was studied. Complexes were prepared with a preparation of EEE IRNA and polylysine of 100,000 molecular weight in a ratio of 1:1. Infectivity was >99% masked. Portions of

TABLE 7. *Effect of heating on recoverable infectivity of stored polylysine-infectious ribonucleic acids (IRNA)*

Treatment	Infectivity (log ₁₀ PFU/ml) ^a	
	Uncomplexed IRNA	Polylysine-IRNA
Untreated.....	5.8	<2.7
Pronase ^b only.....	5.9	4.8
55 C for 2 min + Pronase...	5.9	4.1
75 C for 2 min + Pronase...	5.8	<3.0
100 C for 2 min + Pronase...	5.5	<3.0

^a PFU, plaque-forming units.

^b Conditions for enzyme treatment: 37 C for 45 min; final concentration of Pronase, 1 unit/ml, and of polylysine, 0.2 µg/ml.

complex and of IRNA alone were incubated at 2 to 4 or 37 C for appropriate times and then treated for 30 min at 37 C with Pronase before assay. Starting samples were treated with Pronase without prior incubation. There was no difference in recoverable infectivity between IRNA alone and complexed preparations incubated for 2 hr at 2 to 4 C or for 30 min at 37 C. After 2 hr at 37 C, infectivity recoverable by Pronase treatment of the complex was one-third that of the parallel sample of IRNA alone.

The effect of UV irradiation on polylysine-IRNA was compared with the effect on IRNA alone in studies with IRNA from VEE or EEE virus and polylysine of 100,000 molecular weight. Samples of control IRNA alone or of polylysine-IRNA (polylysine:RNA, 2:1 or 1:1) were exposed, for intervals up to 5 min, to UV irradiation from a General Electric sun lamp at a distance of 32 cm. After Pronase treatment to digest the polylysine, samples were assayed for infectivity. Although it was observed that the inactivation varied quantitatively in the several experiments performed, inactivation of the IRNA in the presence of polylysine was always more rapid than that of the control IRNA alone. Data from one of the studies are shown in Fig. 3. These data, and those from the replicate experiments, indicated that IRNA in the presence of the higher proportion of polylysine was inactivated at a faster rate than that exposed in the presence of the lower proportion of polyamino acid. These differences between effect on IRNA alone and complexed IRNA were observed when temperature of the sample was not controlled during the 5-min exposure and also when the sample container was submerged in an ice-water bath during exposure. Sample temperature rose from

FIG. 3. Effect of ultraviolet irradiation on recoverable infectivity of polylysine-infectious ribonucleic acids [IRNA]. Samples were exposed for appropriate times to irradiation from General Electric sun lamp at a distance of 32 cm. Before infectivity was assayed, samples were treated with Pronase to digest the polylysine. (●), IRNA alone; (▲), polylysine-IRNA, 2:1 (w/w); and (△), polylysine-IRNA, 1:1.

4 to 25°C when uncontrolled and from 4 to 10°C when cooled during irradiation.

We examined methods other than Pronase treatment for their effectiveness in recovering a biologically active IRNA entity from polylysine-IRNA. Methods were chosen because they have been reported effective in denaturing protein or in separating nucleic acid from protein in biological macromolecules or both. Polylysine of 100,000 molecular weight and IRNA of VEE virus were used. For every method tested, equivalent samples of IRNA alone were treated in parallel with polylysine-IRNA. Pronase treatment was effective in recovering infectivity from all of the polylysine-IRNA samples used.

The following treatments failed to effect recovery of any infectivity from polylysine-IRNA despite the fact that such treatments did not significantly denature samples of control IRNA alone: one phenol extraction at 22 or 50°C; 7 M urea (12), pH 4.6; and 1% digitonin (Colon, J. I., and J. B. Idoine, *Bacteriol. Proc.*, p. 159, 1963) or 0.5% deoxycholate (11). Before assay the 7 M urea-treated samples were chromatographed on Sephadex 200 and the pH 4.6 samples on Sepharose 4B. No infectivity was observed in fractions eluted in the range where 90 to 100% of the infectivity was recovered when samples of control IRNA alone were chromatographed.

When polylysine-IRNA was treated once at 50°C with phenol in the presence of approxi-

mately 0.02 M SDS, 0.1% of the original infectivity was recovered. About 10% was recovered by extraction for 5 min at 70°C with 0.05 M or 0.2 M SDS alone (5). Treatment for 1 hr at 22°C with 0.01 M SDS alone effected recovery of about 1%. Parallel treatments with SDS of control IRNA alone yielded 70 to 100% of the original infectivity.

DISCUSSION

The masking of infectivity and protection of IRNA against nuclease inactivation by high molecular weight poly-L-lysine suggest indirectly that complexes are formed between IRNA and the polyamino acid. Results of the sucrose gradient experiments indicate more directly that complexes are formed. The association of polylysine with IRNA was evidenced by different sedimentation patterns for IRNA alone and for polylysine-IRNA. For the latter, the increased infectivity of lower fractions after Pronase digestion was an additional indication of close association between polylysine and the infectious entity.

Mora (7, 8) found that blocking of biological activity by polyelectrolytes (i.e., inhibition of antiserum inactivation of T2 bacteriophage and inhibition of ribonuclease by polyglucose sulfate) were dependent on the electrostatic bonding of polyelectrolyte to enzyme or phage. Our data show that electrostatic bonding is an important factor in the effect of polylysine of 3,000 molecular weight on IRNA, as significant recovery of infectivity was observed when complexes formed with this low molecular weight polyamino acid were suspended in 1 M NaCl. The effects observed with high molecular weight polylysine and IRNA indicate that the bonding in these complexes is more difficult to dissociate since the masking of infectivity by low proportions of polylysine of 100,000 molecular weight was not reversed by treatments with 1 M NaCl, pH 4.6, or 7 M urea. Our observations parallel those of Mora in respect to the dependence on polyelectrolyte nature for effect on biological activity; low molecular weight polylysine was less effective than the high molecular weight material in masking infectivity, and L-lysine when present in 1,000 times the concentration of RNA had no effect.

It is apparent from sucrose gradient centrifugation of polylysine-IRNA that some complexes of IRNA with high molecular weight polylysine remain infectious, i.e., when IRNA alone and complexed IRNA were compared, more infectivity was recovered from rapidly sedimenting material in the case of the complex even without Pronase digestion of polylysine. In this experi-

ment the polylysine-RNA ratio used was low, 1:6, to limit the formation of insoluble complexes. It is possible that masking of infectivity occurs only when some required proportion of polylysine is contained in the complex or that the attached polylysine is capable of masking only when attached in some particular fashion to the IRNA. The structure proposed by Higuchi and Tsuboi (3) for double-stranded RNA and polylysine complexes is a triple helix in which distances between the coils of the RNA helix appear to be restricted by the polylysine. It is possible that high molecular weight polylysine, when complexed with the single-stranded IRNA of VEE or EEE viruses, may limit flexibility of secondary structure and thereby inhibit biological activity. If complexes are able to enter cells, transcription of the viral genome may be prevented in the changed structure. Structural conformation of the IRNA in the complexes may also be responsible for the apparent increased susceptibility of the nucleic acid to inactivation by heat or UV irradiation when it is treated in the complexed state. Loss of infectivity recoverable by Pronase digestion when complexes were stored at 4°C may be explained by increased bonding sites of polylysine and IRNA with time to form enmeshed structures in which the polylysine is no longer available to enzyme action.

At least some of the effect of high molecular weight polylysine on IRNA may be attributed to aggregation. The more rapid sedimentation of complexes in sucrose gradients indicates that some aggregation occurred even with a polylysine-RNA ratio of 1:6. Ultraviolet absorption data on polylysine-IRNA complexes with this ratio showed increased absorption over that of IRNA alone (*unpublished data*) indicating a light-scattering effect attributable to aggregation. Possibly more than one IRNA molecule may attach to a single polylysine chain; such aggregates may exceed a size capable of entering cells.

Whether complexes that we studied are analogous to naturally occurring nucleoproteins and whether the reversible masking of biological activity of nucleic acid shown here parallels natural processes are not disclosed by data presented here. The experiments do suggest that some unmasking of infectivity occurred in vivo

in experimental animals. When 3,500 PFU of IRNA were complexed, no infectivity was detected by plaque assay but 33% mortality occurred in the inoculated chicks. However, for control IRNA the plaque assay is always more sensitive than in vivo tests, e.g., no mortality was demonstrated in chicks with inocula that showed 35 PFU by plaque assay. Complexing with a basic polyamino acid has been shown as a possible mechanism for masking biological activity of IRNA, and restoration of activity was effected by enzymatic digestion of the polyamino acid.

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