Relationship of Molecular Size of $rI_n:rC_n$ (Poly I:C) to Induction of Interferon and Host Resistance (35169)

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The observation that $rI_n:rC_n$ (commonly referred to as poly I:C) from different commercial sources varied considerably in capacity to induce interferon and to protect against virus infections led us to speculate that this variation in activity might be due to differences of molecular weight. The enzymatic synthesis of the homopolymers from nucleoside diphosphates using polynucleotide phosphorylase results in polymers having a wide variation in chain lengths, depending on the conditions of polymerization. The present report describes the relationship of molecular weight of $rI_n:rC_n$ to its physicochemical characteristics and its biological activity. The assays for biological activity included interferon induction and induction of host resistance to viral infection measured *in vivo* and *in vitro*.

Materials and Methods. Polyriboinosinic acid (rI_n) and polyribocytidylic acid (rC_n) were prepared by Dr. J. Zabriskie and Dr. P. Pollak, Merck Process Development Group. Preparation of the polyriboinosinic acid: polyribocytidylic acid ($rI_n:rC_n$) complex was carried out as described previously (1).

Sonic radiation and characterization of $rI_n:rC_n$ preparations. Fragments of $rI_n:rC_n$ were prepared by exposure of 1-mg/ml solutions of the complex for various periods of time to sonic radiation produced by a 20,000 cycle Biosonic III generator equipped with a ¾ in. probe having a 210 W output. All sonic radiation experiments were carried out with cooling. Relative viscosities of $rI_n:rC_n$ solutions were measured using an Ostwald viscosimeter having a flow time of 120 sec with water at 25°. Sedimentation coefficients of $rI_n:rC_n$ solutions were determined by Drs. E. Harris and J. Hirschfield (Merck & Co.) using a Spinco Model E ultracentrifuge equipped with a scanning ultraviolet optical system. Molecular weights were estimated from the empirical equation according to Studier (2) which relates the sedimentation coefficient of DNA directly to molecular weight as follows: mol wt $= 1.15 \times 10^3 \times S^{2.9}_{w,20}$. The assumption is made that the conformation of the double-stranded $rI_n:rC_n$ molecules is similar to double-stranded DNA.

Thermal transition midpoints (T_m) and relative ribonuclease sensitivities were performed as previously described (3). Percentage hyperchromicity was calculated as the percentage increase in optical density measured over a 15° temperature range at 248 mμ. Methods for evaluating the capacity of double-stranded RNA preparations to induce interferon in rabbits and resistance to challenge with pneumonia virus of mice (PVM) *in vivo* and with vesicular stomatitis virus in rabbit kidney cell culture *in vitro* have been previously described (1, 4).

Separation of $rI_n:rC_n$ fragments by chromatography. Chromatography of $rI_n:rC_n$ preparations was carried out on Sepharose 4B (4% beaded agarose) purchased from Pharmacia, Uppsala, Sweden.

One-half ml, containing 7 optical density units (248 mμ) of $rI_n:rC_n$, was applied to a 1.5 × 36-cm column and eluted using 0.006 sodium phosphate, 0.15 *M* NaCl, pH 7 buffer. The void volume of the column was determined using dextran blue 4000 which was partially excluded from the gel. Average molecular weights of the chromatographed $rI_n:rC_n$ preparations varied from 7.8×10^6 for undegraded $rI_n:rC_n$ to 2.8×10^5 for sonic treated $rI_n:rC_n$ as calculated from the cor-

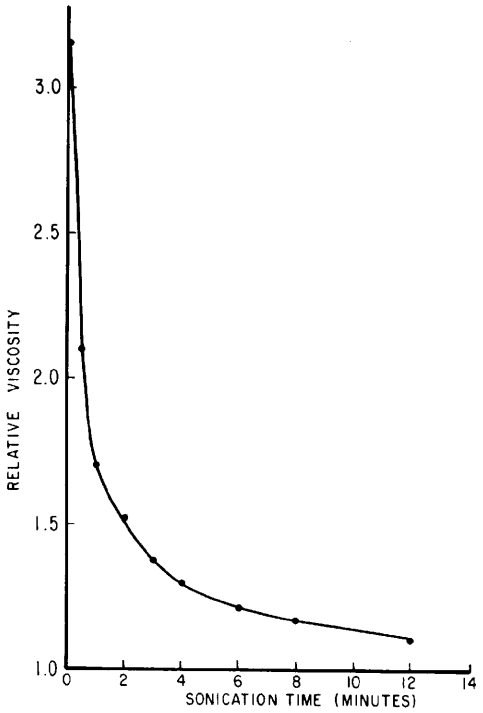

FIG. 1. Effect of sonic treatment on relative viscosity of rI_n:rC_n solutions.

responding sedimentation coefficients of 21 to 6.7.

Results. Effect of sonic radiation on physical characteristics of solutions of rI_n:rC_n. Figure 1 shows that exposure of a solution of rI_n:rC_n of high molecular weight to sonic radiation for increasing time periods caused exponential decrease in its relative viscosity. Exposure for 1 min decreased the relative viscosity from 3.2 to 1.7 or 68% of the potential decrease to 1.0 (the relative viscosity of water). The data in Table I illustrate that the decrease in viscosity was accompanied by cleavage of the high molecular weight rI_n:rC_n to complexes of lower molecular weight. Progressive decrease in molecular weight was accompanied by progressive reduction in the thermal transition midpoint (T_m), progressive decrease in hyperchromicity on thermal dissociation, and progressive increase in sensitivity to degradation by pancreatic ribonuclease. These alterations were due apparently to decrease in the average chain length of the complexed rI_n:rC_n rather than to an increase in the amount of uncomplexed homopolynucleotides since, as shown in Fig. 2, the untreated and the sonic-treated rI_n:rC_n had equivalent ultraviolet absorption spectra. However, the sonic-treated rI_n:rC_n "melted" over a broader temperature range, showed a lower T_m, and gave a smaller hyperchromic effect than the high molecular weight polynucleotide (see Fig. 2).

Effect of sonic radiation on capacity of solutions of rI_n:rC_n to induce interferon and host resistance in vivo and in vitro. A preparation of rI_n:rC_n was treated by sonic radiation for 16 min and the irradiated and control samples were tested for the capacity to induce interferon in rabbits and resistance to viral infection in cell culture. Table II shows

TABLE I. Characterization of rI_n:rC_n Degraded by Sonication.

Relative viscosity	$S_{w,20}$	Mol wt[a]	T_m (°C)	Hyperchromicity[b] (%)	Relative RNase[c] sensitivity	$E_{1\%}$ (265 mμ)
3.9	21.1	7.8×10^6	63.5	71.2	100	140
2.1	16.9	4.2×10^6	62.5	70.7	114	140
1.7	13.7	2.3×10^6	62.0	71.7	115	139
1.4	11.1	1.2×10^6	62.5	67.0	117	140
1.3	9.3	7.4×10^5	61.5	67.0	129	139
1.2	8.5	5.6×10^5	61.0	62.0	143	139
1.1	7.9	4.6×10^5	60.0	59.0	147	143
1.05	6.7	2.8×10^5	58.5	49.0	220	145

[a] $MW = 1.15 \times 10^3 \times S_{w,20}^{2.9}$.

[b] Percentage increase in OD during thermal dissociation (T_m).

[c] Increase (OD units/min) during pancreatic ribonuclease hydrolysis. Rate for undegraded rI_n:rC_n was set at 100.

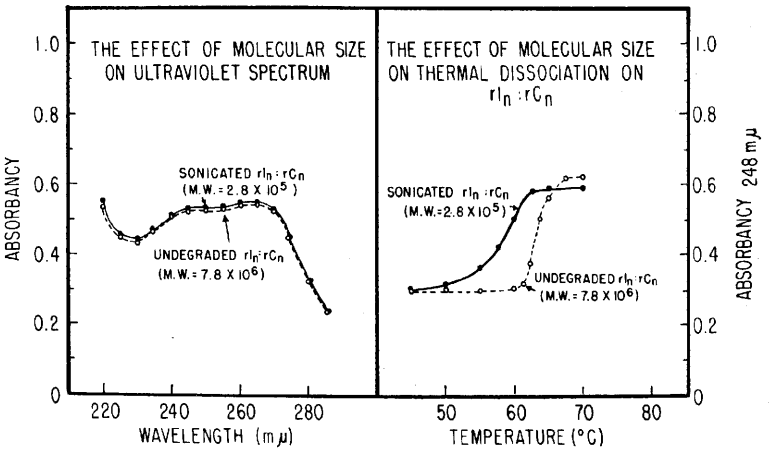

FIG. 2. Effect of molecular size on ultraviolet absorption spectrum and thermal dissociation of $rI_n:rC_n$.

that sonic radiation caused only slight, if any, diminution of capacity to induce interferon even though the relative viscosity of the solution was lowered from 3.9 to 1.05. The capacity to induce resistance to virus in cell culture was reduced from 2- to 4-fold.

Table III shows that there was progressive diminution in the capacity of $rI_n:rC_n$ to induce resistance to PVM virus infection in mice with reduction of viscosity by sonic radiation. Untreated $rI_n:rC_n$ with relative viscosity of 3.9 afforded 94, 80, and 60% excess survival against PVM virus in mice given 4, 1, and 0.25 μg, respectively. By contrast, these same doses of polynucleotide treated for 12 min to reduce the relative viscosity to 1.05 gave only 24, 12, and 20% protection, respectively. The values obtained in tests with $rI_n:rC_n$ of intermediate viscosity generally lay in between and were roughly proportional to the viscosity.

Fractionation of sonic-radiated $rI_n:rC_n$ solutions by chromatography on Sepharose 4B. The various molecular species of $rI_n:rC_n$ which resulted from sonic radiation were readily resolved by chromatography on agarose (Sepharose 4B) as shown in Fig. 3. The major portions of the polynucleotide complex which had been reduced to a relative viscosity of 1.3 or less were eluted from the

TABLE II. Effect of Sonic Radiation on Capacity of $rI_n:rC_n$ to Induce Interferon in Rabbits and Interference in Primary Rabbit Kidney Cell Cultures.

$rI_n:rC_n$		Assay		
		Induction of interferon in rabbits		Interference in cell cultures; minimal effective dose (μg/ml)
Sonic radiation time (min)	Relative viscosity	Dose (μg/rabbit)	Interferon titer in individual rabbit sera[a]	
0	3.9	5	>640, 320	0.002–0.004
		1	160, 320	
16	1.05	5	>640, 320	0.008
		1	5, 80	
—		0 (control)	<5, <5	

[a] The sera were collected 2 hr after administration of $rI_n:rC_n$ and were tested for interferon by the tube dilution method in primary rabbit kidney cells using vesicular stomatitis virus for challenge.

TABLE III. Capacity of Sonic-Treated $rI_n:rC_n$ to Induce Resistance Against Pneumonia Virus Infection of Mice (PVM).

Relative viscosity	Approx mol wt[a]	Total dose/mouse (μg)[b]	PVM test % excess survival[c]	Excess survival day[d]
3.9	7.8×10^6	4	94	>7
		1	80	>7
		0.25	60	>7
2.1	4.2×10^6	4	90	>7
		1	75	>7
		0.25	60	>7
1.7	2.3×10^6	4	47	>7
		1	55	>7
		0.25	20	3
1.4	1.2×10^6	4	75	>7
		1	42	6
		0.25	20	2
1.3	7.4×10^5	4	50	>6
		1	45	>6
		0.25	5	1
1.1	4.6×10^5	4	30	5
		1	15	3
		0.25	5	1
1.05	2.8×10^5	4	24	3
		1	12	2
		0.25	20	2

[a] These molecular weights are approximations derived using Studier's equation.

[b] Mice were pretreated with 0.03 ml of $rI_n:rC_n$ solutions intranasally 3 hr prior to intranasal infection with 30 LD_{50} of PVM (20 mice were used/point).

[c] Percentage survival corrected for survival in untreated controls (60 mice were used in the control group).

[d] Mean survival day in excess of mean survival day of untreated controls.

columns at volumes (V_e) which were in excess of the void volume (V_o).

Plotting the V_e/V_o ratio of the various Sepharose fractions against average molecular weight, as determined by sedimentation analysis, made possible the construction of the curve shown in Fig. 4 whereby approximate molecular weights of fractions could be determined based on V_e/V_o ratio alone. A number of such fractions which were separated on Sepharose and the molecular weights determined by V_e/V_o ratio were assayed for capacity to induce interferon in rabbits and interference against VSV in primary cell cultures of rabbit kidney. The findings given in Table IV showed that $rI_n:rC_n$ fractions which had an approximate average molecular weight of 1.2×10^5 or greater were roughly as active in inducing interferon and resistance to viral infection as was the untreated complex. Fractions with lower molecular weight displayed significantly reduced activity.

Discussion. Systematic investigation of the factors which influence the capacity of polynucleotides to induce interferon and host resistance has been pursued in our laboratories since the initial observation by our group (1, 4–7) of the activity of the double-stranded RNA's. The requirement of double-strandedness and the ribose sugar moiety for induction has been confirmed by numerous investigators (8–11).

The present report describes the influence of molecular weight of the complexed $rI_n:rC_n$ on its physical properties and on its capacity to induce interferon and resistance to viral infections *in vivo* and *in vitro*. Decrease in the molecular weight of the $rI_n:rC_n$ was accompanied by decrease in thermal stability and increased sensitivity to degradation by pancreatic RNase. It appeared that the capacity of $rI_n:rC_n$ to induce resistance to infection with pneumonia virus of mice

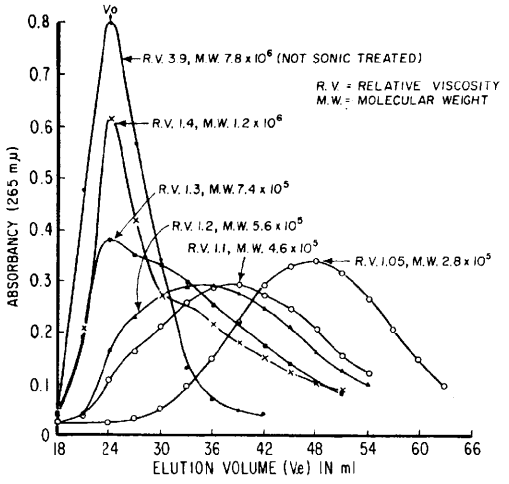

FIG. 3. Chromatography of sonic degraded $rI_n:rC_n$ on agarose (Sepharose 4B).

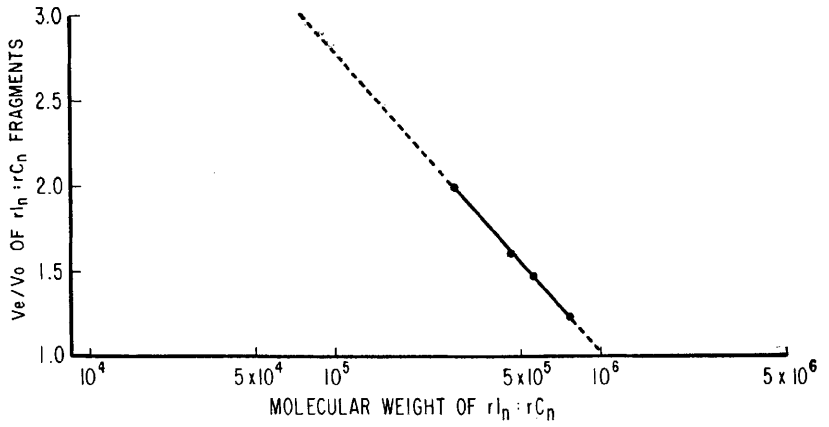

Fig.

FIG. 4. Calibration curve of sepharose 4B column: molecular weight determination of $rI_n:rC_n$ fragments.

was more sensitive to reduction in molecular weight than was either the capacity to induce interferon in rabbits or resistance to VSV infection in cell culture. Whether the decrease in these biological activities resulted from the decreased bonding energy between the homopolynucleotides or the increased sensitivity to enzymatic destruction of the complex remains unanswered.

Double-stranded RNA's of synthetic or natural origin with greatly different thermal stabilities have been shown (10, 12) to have comparable interferon inducing capacities. It would appear, therefore, that thermal stability plays only a minor role in determining the inductive capacity of double-stranded RNA beyond a minimal thermal stability level which is sufficient to maintain double-strandedness under physiological conditions. RNase susceptibility of the double-stranded RNA may be more important, varying in extent in different biological test systems. It may well be that inductive capacity will only be influenced beyond a minimal RNase sensitivity level as seems true for the minimal thermal stability level.

TABLE IV. Capacity of Fractions of Sonicated $rI_n:rC_n$ from Sepharose Chromatography to Induce Rabbit Serum Interferon and Interference in Primary Rabbit Kidney Cell Cultures.[a]

Sepharose fractions	$\frac{V_e}{V_0}$	Estimated mol wt	Dose/rabbit (μg)	Rabbit serum interferon titer	Interference in cell culture % of activity relative to V_0 fraction
25	1.0	$>10^6$	2	80, 160	100
33	1.38	6.4×10^5	2	640, 160	—
40	1.66	4.4×10^5	2	320, <5	200
48	2.0	2.8×10^5	2	20, 80	100
54	2.25	2.0×10^5	2	20, 20	100
63	2.63	1.2×10^5	2	<5, 160	—
66	2.75	10^5	—	—	50
70	3.0	7.0×10^4	2.8	<5, <5	—
74	3.1	6.0×10^4	—	—	12.5
Control	—	—	—	<5, <5	—

[a] V_e/V_0 = elution volume of fraction/void volume; V_0 fraction represents high molecular weight $rI_n:rC_n$ excluded from the Sepharose 48 column; 2 rabbits were used/point.

A separate report (13) will present the findings in a study to determine the importance of molecular size of each of the homopolynucleotides in the activity of the $rI_n:rC_n$ complex.

Summary. Preparations of high molecular weight $rI_n:rC_n$ were exposed to sonic radiation. A decrease in the average molecular weight of the polynucleotide complexes after treatment was accompanied by an exponential decrease in viscosity of the preparation, an increase in the sensitivity of the polynucleotide complex to degradation by pancreatic ribonuclease, a decrease in the thermal transition midpoint, and a decrease in the hyperchromicity observed during thermal transition.

The lower molecular weight $rI_n:rC_n$ complexes were tested for their capacity to induce interferon in rabbits and resistance against VSV infection in cell culture and PVM infection in mice. Sonic-treated preparations of $rI_n:rC_n$ reduced to an average molecular weight of approximately 4.6×10^5 were markedly reduced in capacity to protect mice against PVM infection, compared with untreated $rI_n:rC_n$ preparations containing complexes having an average molecular weight of approximately 7.8×10^6. The capacity of sonic-treated $rI_n:rC_n$ to induce production of rabbit interferon and resistance to virus infection in cell culture was more resistant to molecular weight reduction. Significant decrease in activity was observed in $rI_n:rC_n$ fractions with approximate molecular weights less than 1.2×10^5.

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