INDUCERS OF INTERFERON AND HOST RESISTANCE, I. DOUBLE-STRANDED RNA FROM EXTRACTS OF PENICILLIUM FUNICULOSUM

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Since interferon[1] *per se* shows little promise as a prophylactic or therapeutic agent, interest has shifted to a search for acceptable inducers by which the body might be stimulated to make its own interferon.

Shope[2] demonstrated that a substance which was derived from *Penicillium funiculosum* and which was called helenine induced resistance to Semliki Forest and to Columbia SK virus infections in mice. Lewis *et al.*[3] fractionated helenine and prepared a factor active in the mouse assay which exhibited properties of a nucleoprotein. Rytel, Shope, and Kilbourne[4] demonstrated that helenine elicited a viral inhibitor in cell cultures and in mice which exhibited properties similar to those of interferon. In commenting, they stated that "the active principle in helenine is still a matter of conjecture since it has not been obtained in a sufficiently pure form to permit definite identification."

Studies in our laboratories during the past several years have been directed toward the discovery of an interferon inducer which would be worthy of clinical evaluation. It was found that certain ribonucleic acids were highly active in inducing interferon and host resistance but were dependent upon (*a*) freedom from inhibitory protein and (*b*) multistrandedness of the RNA. Ribonucleoproteins and single-stranded nucleic acids were inactive.

This series of papers describes the purification or synthesis and characterization of three kinds of multistranded RNA active in inducing interferon and host resistance. The present report describes the isolation and characterization of a double-stranded RNA from extracts of *Penicillium funiculosum* (helenine) which, when freed of protein, is a highly active inducer of interferon and host resistance. The substance is referred to as HeI-RNA. Subsequent papers will present data showing high level interferon and host resistance-inducing activities of multistranded complexes of synthetic polynucleotides[5] and of double-stranded RNA of reovirus origin.[6]

Materials and Methods.—(1) *Extract of Penicillium funiculosum:*[7] *P. funiculosum* was grown as described elsewhere.[3] The mycelium was extracted with 0.033 *M* sodium phosphate buffer, pH 8.0, by rapid stirring for 1 hr at room temperature. The clarified extract was treated as shown in steps A–C of Table 1. The supernate served as starting material for fractionation and purification purposes.

(2) *Assay for interferon induction in rabbits:* Young adult New Zealand white rabbits weighing 4–5 lb were injected intravenously with 0.5 ml of the material for assay. Blood samples were taken 2 hr later. The sera were assayed for interferon content in primary rabbit renal cell cultures in roller tubes or in plaque assay flasks by published procedures[8, 9] using vesicular stomatitis virus for challenge. The titer of interferon was the highest initial dilution of rabbit serum which showed 100% suppression of viral cytopathic effect in 50% or more of the tube cultures, or which effected at least 50% reduction in plaques compared with controls in the plaque assay. The plaque assay was used routinely for the studies connected with the chemical and physical charac-

terizations and for the host specificity test. The roller tube culture assay was employed in the biological determinations.

(3) *Assay for induction of host resistance in mice:* The procedures for measuring induction of resistance in mice to Columbia SK and pneumonia virus of mice (PVM viruses) have been described elsewhere.[3, 10] Protective effect was expressed in terms of average survival time and per cent survival for 10 or 14 days following virus challenge, compared with untreated controls.

(4) *Chemical assays:* Base composition was determined by paper chromatography following perchloric acid hydrolysis.[11] Ribose was determined by the method of Mejbaum,[12] and polysaccharide content by the method of Dische.[13] Protein assays were performed by the method of Lowry *et al.*,[14] and phosphorus analyses were made by a modified method of Allen.[15] Deoxyribose content was determined according to the method of Burton.[16]

Results.—(1) *Isolation of RNA inducer from P. funiculosum (HeI-RNA):* Studies of induction of host resistance by helenine revealed a considerable variation in amount of activity from batch to batch.[3] Similar variation was noted also for interferon induction in the present studies until it was shown that the inducer was actually present in essentially all lots but that its activity was masked or depressed by presence of substances which could be removed by treatment with phenol. Such phenolic treatment permitted separation of the biologically active RNA which was then purified further as outlined in steps D–G of Table 1.

Table 2 shows the removal of inhibitor from the HeI-RNA.

The data relating to chromatographic purification of the phenol-treated extract are shown in Figure 1. The ecteola-cellulose was suspended successively in 0.5 *M* NaOH, distilled water, 0.5 *M* NaH_2PO_4, and 0.01 *M* sodium phosphate buffer at pH 7.0. The slurry was introduced into the column and was washed further with 200 ml of 0.01 *M* sodium phosphate buffer solution. Phenol-treated extract (110 ml), representing a 25-fold concentrate of the aqueous extract of the acetone precepitate, was applied to the column. The column was eluted with NaCl gradient in 0.01 *M* sodium phosphate buffer solution, pH 7.0, as shown in Figure 1. An inactive polysaccharide was present which was not adsorbed to the column and which was removed by the primary washing with the buffer solution. The peak activity was

TABLE 1

STEPS IN PURIFICATION OF HeI-RNA FROM *P. funiculosum* EXTRACT

A. Add 1 vol of acetone to mycelial extract.
B. Extract precipitate with distilled water, dialyze vs. saline solution, and centrifuge 18 hr at 35,000 × *g*.
C. Resuspend pellet in 0.01 *M* sodium phosphate buffer, pH 7.0, and clarify by centrifuging 10 min at 2,000 rpm.
D. Add equal volume of 88% phenol to supernate and stir 30 min at 35–40°C. Clarify by centrifugation at 2000 rpm for 30 min at 5°C.
E. Extract the aqueous layer twice more with phenol at 25°C for 30 min. Clarify by centrifuging at 2000 rpm for 30 min at 5°C.
F. Dialyze supernate against 50–100 vol of 0.01 *M* sodium phosphate buffer, pH 7.0.
G. Chromatograph twice on ecteola-cellulose employing sodium chloride gradient elution.

TABLE 2

REMOVAL OF INHIBITOR OF HeI-RNA BY TREATMENT WITH PHENOL

Dilution of *P. funiculosum* extract	Interferon Titers in Sera of Individual Rabbits* Given— Untreated extract	Phenol-treated extract
1:2	5, 10	640, 640
1:4	40, 40	640 or >, 640 or >
1:8	40, 40	160, 160
1:16	5, 10	80, 20

Interferon titers are expressed as reciprocal of serum dilution.
* The sera from the two uninoculated control rabbits gave inhibitory titers of 5.

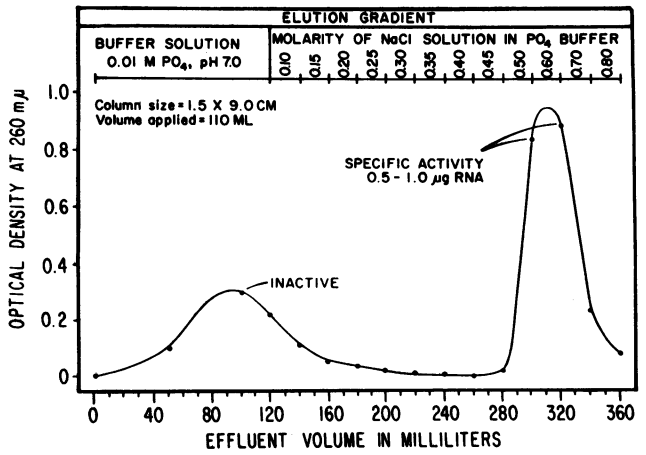

FIG. 1.—Primary chromatography on ecteola-cellulose of phenol-treated extract of *P. funiculosum*.

eluted with 0.5–0.6 *M* NaCl. This contained RNA which induced interferon to a titer of 1:20 or greater in rabbits given 0.5–1.0 µg dose. The peak fractions of active substance were pooled, dialyzed against 0.01 *M* sodium phosphate buffer, pH 7.0, and 100 ml was rechromatographed on ecteola-cellulose in the manner described above. The specific activity for rabbits was 0.25–0.5 µg RNA.

(2) *Interferon induction by HeI-RNA compared with RNA and DNA of diverse origin:* A graded dose response assay to measure the activity of the peak fractions of HeI-RNA obtained following chromatography was carried out and the results are shown in Table 3. As little as 0.125 µg of HeI-RNA, calculated on the basis of an extinction coefficient ($E^{1\%}_{260}$) of 210, sufficed to induce interferon in rabbits. A number of single-stranded RNA's and a double-stranded DNA which are also listed in Table 3 were tested for comparative purpose. None was found active. Additional tests *in vitro* in chick embryo fibroblasts showed lack of inducing activity.

(3) *Characterization of interferon induced in rabbits by HeI-RNA:* The viral inhibitory substance in the sera of rabbits injected with HeI-RNA was identified as

TABLE 3

INTERFERON-INDUCING CAPACITY IN RABBITS OF HeI-RNA COMPARED WITH THAT OF RNA AND DNA OF DIVERSE ORIGIN

Source and kind of nucleic acid	Chemical nature	Intravenous dose per rabbit (µg)	Interferon titer of rabbit serum
HeI-RNA	Double-stranded RNA	8	80, 640 or >
		2	80, 160
		0.5	20, 40
		0.125	5, 10
		0.0	<5, <5
Newcastle disease virus virion RNA[a]	Single-stranded	10	<5
Influenza virus virion (WS) RNA[a]	" "	10	<5
Tobacco mosaic virus virion RNA[b]	" "	40	<5
Yeast ribosomal RNA[c]	" "	1000	<5
Yeast sRNA[d]	" "	200	<5
Yeast core RNA[e]	" "	100	<5
Mouse liver (ribosomal) RNA[e]	" "	200	<5
Bovine liver sRNA[f]	" "	200	<5
Calf thymus DNA[c]	Double-stranded	200	<5

[a] Prepared by the method of Sokol *et al.*[17] [b] Obtained from Dr. Wendell Stanley. [c] Worthington Biochemicals, Freehold, New Jersey. [d] General Biochemicals, Chagrin Falls, Ohio. [e] Prepared according to Barlow *et al.*[18] [f] Sigma Chemicals, St. Louis, Missouri.

TABLE 4

SPECIES-SPECIFICITY OF INTERFERON INDUCED IN RABBITS BY INTRAVENOUS INJECTION OF HeI-RNA

Expt. no.	Interferon Titer Based on Plaque Assay in Cell Cultures: Homologous Cells: Rabbit kidney	Heterologous Cells: Mouse embryo	Heterologous Cells: Chick embryo
1	160	<20	ND*
2	96	<12	<16

* Not done.

TABLE 5

TRYPSIN SENSITIVITY OF INTERFERON IN SERUM OF RABBITS INDUCED BY INTRAVENOUS INJECTION OF HeI-RNA

Treatment	Interferon Titer: Expt. 1	Expt. 2
None (control)	10	52
*Trypsin (50 μg/ml)	<10	<20

* Crystalline, 4 hr at 35°C.

interferon based on its biological and chemical properties. (*a*) *Host species-specificity* (Table 4) shows that resistance to the vesicular stomatitis virus was evoked in the homologous rabbit kidney but not in heterologous mouse and chick embryo cell cultures. (*b*) *Trypsin sensitivity* (Table 5) shows that the rabbit serum interferon activity was destroyed by trypsin. (*c*) The *molecular weight* of interferon in the rabbit serum induced by HeI-RNA was determined by filtration through Sephadex G-200 columns. Molecular weight was calculated according to the method developed by Squire.[19] There were two species of interferon present and the molecular weights were calculated to be 61,000 and 130,000. (*d*) The *isoelectric point* of the HeI-RNA-induced rabbit interferon was measured based on elution curves from CM-Sephadex according to a previously published procedure.[8] The fraction with peak interferon activity had pH's of 6.5 and 6.7 in two experiments and the isoelectric point was calculated to be 6.9–7.1.

(4) *Induction of host resistance to viral infection by HeI-RNA:* The HeI-RNA induced resistance to both PVM and to Columbia SK viruses in mice. Table 6 shows that both survival time and per cent survival of the mice were greatly enhanced by administration of the RNA.

(5) *Physical and chemical characterization of the HeI-RNA:* (a) The *ultraviolet absorption spectrum* of the purified HeI-RNA was typical for nucleic acid with minimum at 230 mμ and maximum at 257 mμ. The 260:230 ratio was 2.30 and the 260:280 ratio was 2.18.

(b) *Chemical composition:* The HeI substance contained 38% ribose, 8.4% phosphorus, plus purine and pyrimidine bases. The values for ribose and phosphorus approximate the theoretical values for RNA. Protein, polysaccharide, and deoxyribose were absent. R_f values obtained for the bases were in close agreement with published values[20] for the same system and established the presence of adenine, guanine, cytosine, and uracil which are normally found in ribonucleic acids.

(c) *Sensitivity of biological activity of HeI-RNA to RNase, DNase, sodium periodate,*

TABLE 6

INDUCTION OF RESISTANCE IN MICE TO VIRAL INFECTION BY HeI-RNA

Virus challenge	Total RNA dose per animal	No. survived/ total infected	Survival (%)
PVM	20 μg*	18/20	90†
"	0 (control)	0/30	0
Columbia SK	50 μg‡	11/15	73†
"	0 (control)	2/60	3.3

PVM (pneumonia virus of mice).
* HeI-RNA given intranasally in 0.03 ml amount 18 hr prior to 50 LD_{50} of PVM virus challenge.
† Survivors at 14 and 10 days, respectively. Controls had mean survival time of 7 and 5 days, respectively.
‡ HeI-RNA given intraperitoneally in divided dose 18 hr prior to and 3 hr after challenge with 30 LD_{50} of Columbia SK virus.

TABLE 7

FINDINGS IN TESTS OF SENSITIVITY OF HeI-RNA TO VARIOUS TREATMENTS

Treatment of HeI-RNA*				
Substance	Concentration	Temp. (°C)	Time	Titer of interferon induced in rabbits
Ribonuclease	0.2 μg/ml	25	30 min	320
Untreated control	—	"	"	320, 640
Ribonuclease	20 μg/ml	56	2 hr	<5
Untreated control	—	"	"	640
Deoxyribonuclease†	5 μg/ml	25	1 hr	80, 160
Untreated control	—	"	"	80, 160
Sodium periodate	0.01 *M*	"	"	40, 640 or >
Untreated control	—	"	"	40, 320
Formalin	1.5%	35	4 hr	640 or >
Untreated control	—	"	"	320, 640
Nothing (control)	—	—	—	<5, 5

* Treated at concentration of 16 μg RNA/ml.
† Crystalline; Worthington Biochemicals, Freehold, New Jersey.

and formaldehyde: HeI substance was tested under the conditions shown in Table 7. Ribonuclease at 20 μg/ml at 56°C destroyed the biological activity. The interferon inducing activity was resistant to DNase, periodate, and formaldehyde.

(d) *Relative resistance of HeI-RNA to ribonuclease:* HeI-RNA was treated with RNase at pH 7.0 and the resulting degradation of the RNA was followed by measuring the increase in optical density at 260 mμ with a Beckman DB-G recording spectrophotometer equipped with a controlled heater compartment. The conditions are indicated in Figure 2. The HeI-RNA was resistant to RNase at 25°C at the 0.2 μg/ml level and increase in temperature and RNase concentration was necessary to effect partial enzymatic degradation. However, as shown in Figure 3, degradation by RNase at the 0.2 μg/ml level at 25°C was achieved when the HeI-RNA was previously denatured by heating for five minutes at 110°C and cooled rapidly to prevent renaturation.

(e) *Reaction of HeI-RNA with formaldehyde:* HeI-RNA and yeast ribosomal RNA were incubated at 35°C for four hours in presence or absence of 1.5 per cent formaldehyde. Figure 4 shows only small change in the ultraviolet spectrum of HeI-RNA following treatment with formaldehyde. By contrast, there was consider-

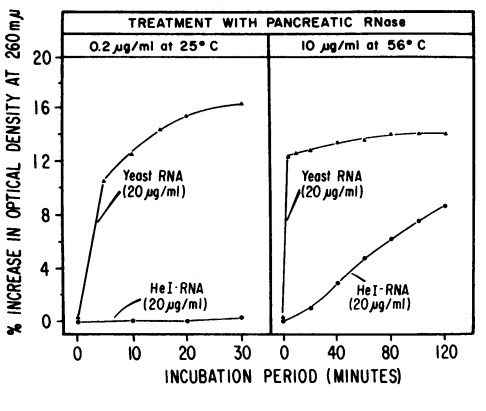

FIG. 2.—Comparative rates of degradation of HeI-RNA and of yeast RNA by RNase.

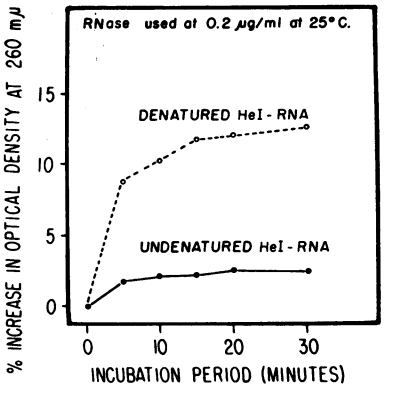

FIG. 3.—Effect of heat denaturation on rate of degradation of HeI-RNA by RNase.

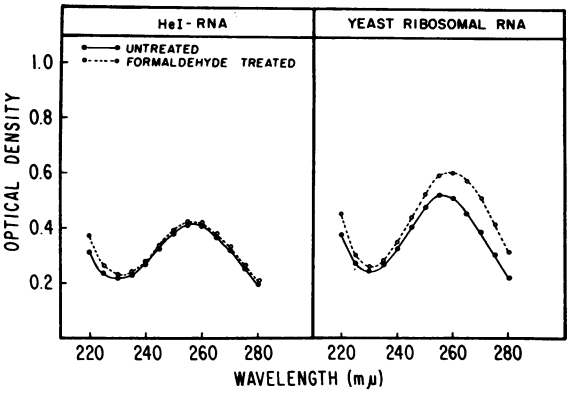

FIG. 4.—Effect of incubation with 1.5% formaldehyde for 4 hr at 35°C on ultraviolet absorption spectra of HeI-RNA and of yeast ribosomal RNA.

able increase in absorbancy of yeast RNA with a shift in the absorbancy maximum to a slightly longer wavelength. These data were interpreted to show relatively fewer free amino groups in the HeI-RNA than in the yeast RNA, and to indicate a highly ordered secondary structure such as a double-stranded helix.[21]

(f) *Thermal denaturation* of HeI-RNA was measured in experiments performed using a Beckman DB-G spectrophotometer equipped with a *Tm* analyzer and recorder according to details shown in Figure 5 *A* and *B*. The increase in absorbancy of the double-stranded HeI-RNA at 260 mμ shown in Figure 5*A* was a function of temperature at two different salt concentrations. In SSC (0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7.0) there was only small increase in absorbancy even at 100°C, indicating that the *Tm* (thermal transition mid-point) was higher than this figure. At the lower ionic strength (0.1 SSC), hyperchromicity of 32 per cent occurred principally in the range of from 85 to 100°C with a *Tm* of 95°C. Single-stranded RNA from yeast ribosomes showed lesser hyperchromicity (20%) and this was over a broad temperature range between 40 and 75°C (*Tm* 55°C) in SSC. These findings showed that HeI-RNA has a high level of thermal stability. Figure 5*B* shows that formaldehyde in a concentration of 2.76 per cent effected a 44 per cent increase in absorbancy of HeI-RNA in SSC on heating to 95°C. Haselkorn and Doty[22] have demonstrated that formaldehyde reduces the *Tm* of hydrogen-bonded helical polynucleotides.

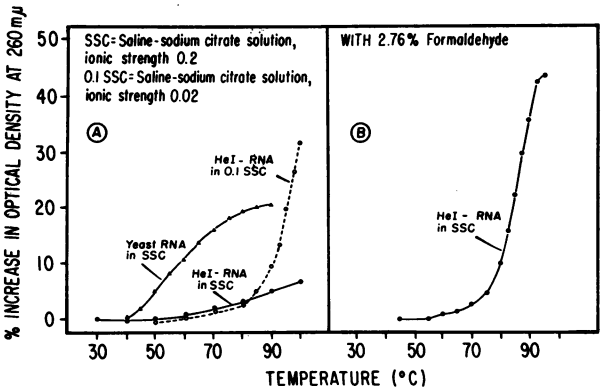

FIG. 5.—(*A*) Thermal transition curves of HeI-RNA in SSC, 0.1 SSC, and yeast RNA in SSC. (*B*) Thermal transition curve of HeI-RNA in SSC containing 2.76% formaldehyde solution.

(g) The *sedimentation coefficients* for three preparations of HeI-RNA at a concentration of 70 μg/ml were determined in a Spinco model E analytical centrifuge. The $s_{20,w}$ values were 10.8, 12.6, and 12.9 with an average of 12.1. The molecular weight of HeI-RNA may be calculated to be about 1.6×10^6 if the relationship $s_{20,w} = 0.0882\ M^{0.346}$ suggested by Studier[23] for native DNA is true also for double-stranded RNA.

Discussion.—The present report describes the isolation from extracts of *Penicillium funiculosum* of a highly purified double-stranded RNA designated HeI-RNA which was active in microgram amounts in inducing interferon in rabbits injected intravenously and which induced host resistance in mice to infection with Columbia SK and PVM viruses. Double-strandedness of HeI-RNA was established based on high thermal transition temperature (>100°C),[21, 24] reduction in thermal transition temperature by low ionic strength[22] and by formaldehyde,[21, 22] and resistance to RNase at room temperature under conditions where ribosomal RNA is rapidly degraded. Treatment with higher concentrations of RNase at elevated temperature resulted in partial degradation (as observed by hyperchromicity) and loss of biological activity. Thermal denaturation of the RNA by heating at the critical temperature and prevention of renaturation resulted in loss of biological activity and greatly increased susceptibility to RNase. Resistance to formaldehyde at 35°C is reflected by a lack of shift in absorption maximum and retention of biological activity.[21]

Isaacs[25] reported induction of interferon in cell culture by foreign RNA and by chemically altered homologous RNA but subsequently questioned[26] the validity of his nucleic acid hypothesis. He stated that large amounts of RNA were needed (100 μg/cell culture) and the titer of inhibitor was very low. All the ribonucleic acids he tested were single-stranded. The present report establishes the lack of interferon-inducing capacity of such single-stranded RNA. Further, present findings as well as those recorded by others[1] have failed to confirm interferon induction by many ribo- and deoxy-ribonucleic acids, by nucleosides, and by nucleotides used at high concentrations as reported by Jensen *et al.*[27–29]

The essential element in induction by HeI-RNA is double-strandedness and one might reasonably question why double-stranded RNA should be present in mold mycelia since double-stranded RNA is a replicative form such as found in viral infection.[30] A possible explanation for this is that the *P. funiculosum* culture might have carried a hypothetical viral infection.

Subsequent reports in the present series confirm the unique requirement of polystranding of RNA for interferon induction as it relates to certain synthetic polynucleotides and to RNA of reovirus origin. The high-level activity in microgram amount and the apparent lack of toxicity have provided a basis for attempted application of these substances to treatment of viral infection in man.

Summary.—A highly purified double-stranded RNA termed HeI-RNA which was active in microgram amount in inducing interferon and resistance to virus infection *in vivo* and *in vitro* was recovered from extracts of mycelium of *Penicillium funiculosum*. DNA and single-stranded RNA were inactive. Details relating to the biological, chemical, and physical properties of the RNA and the interferon induced by it are presented. The finding of the requirement of double-strandedness for activity is new and adds to the understanding of some of the requirements for induction of interferon and host resistance by ribonucleic acid.

The sedimentation analyses were carried out by R. Ziegler. Valuable technical assistance was given by J. Liedtke, K. Young, J. Armstrong, C. Galullo, and C. Bonoma.

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