and the significance of these findings has recently been discussed by Nii *et al.* (*18*). When sucrose gradient rate-zonal centrifugation of the frog HTV was followed by isopycnic banding of the virus zones, the nuclear enveloped particles banded at density 1.20 to 1.21.

The results of our zonal fractionation–embryo injection study suggest that this enveloped form of the frog herpes-type virus plays a role in the genesis of the Lucké tumor.

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17. The term "intranuclear sac" was used by Lunger *et al.* (*10*), who noted that the sac membrane was fused with the inner nuclear membrane. Virus particles within these intranuclear sacs probably lie between the inner and outer nuclear membranes. In this report the use of the term "nuclear sac" merely implies that in thin section these sacs of virus are surrounded by nucleoplasm rather than cytoplasm (see Fig. 2A, *ins*); and the term "nuclear enveloped form" refers to particles that are morphologically similar to the enveloped form found within these intranuclear sacs; that is, they are invested with a thick envelope that is closely applied to the capsid (see Fig. 2B).
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Interferon Induction Increased through Chemical Modification of a Synthetic Polyribonucleotide

Abstract. *The alternating copolymer riboadenylic-ribouridylic acid gained a significant increase in ability to stimulate interferon production (2- to 20-fold) and cellular resistance (100- to 10,000-fold) both in vitro and in vivo upon substitution of phosphate by thiophosphate groups. The resulting nucleotide analog was also 10 to 100 times less sensitive to degradation by pancreatic ribonuclease, as determined by residual antiviral activity.*

Various synthetic polyribonucleotides stimulate interferon production both in vitro and in vivo (*1–4*). A stable, highly ordered, hydrogen-bonded (*3*)—hence double- or multistranded (*1*)—secondary structure seems to be an essential requirement for their antiviral activity. The reason for the greater activity of stable, double- or multistranded complexes is not known but might be related to either increased inducer penetrability, better interaction with a specific receptor site, or resistance to premature enzymatic digestion. Chemical modification of the riboadenylic-ribouridylic acid copolymer [poly r(A-U)] to a nucleotide analog [poly r(AS-US)] in which each phosphate group was replaced by a thiophosphate group results in a significant decrease of the rate of breakdown by several enzymes (spleen phosphodiesterase, snake venom phosphodiesterase, micrococcal nuclease, and pancreatic ribonuclease) (*5*). This increased resistance to enzymatic degradation is associated with an increase of antiviral activity.

The antiviral activity of poly r(AS-US) was compared to poly r(A-U) and the homopolymer pairs polyriboadenylic

Table 1. Comparative study of antiviral activity of poly r(AS-US), poly r(A-U), (poly rA)•(poly rU), and (poly rI)•(poly rC) in human skin fibroblasts.

Polymer	Cellular resistance minimal inhibitory concentration* (μg/ml)		Interferon production† (unit/4 ml)		Thermal stability-T_m (°C)	
	Preparation 1	Preparation 2	Preparation 1 (40 μg/ml)	Preparation 2 (8 μg/ml)	In saline (0.15*M* Na^+)	In 0.01*M* citrate buffer (0.01*M* Na^+)
r(AS-US)	0.001	0.0001	62	24	N.T.‡	48
r(A-U)	0.1	1.0	26	⩽1	69.5§	48(*9*)
(poly rA)•(poly rU)	0.2	N.T.	⩽1	N.T.	57.5(*3*)	38(*10*)
(poly rI)•(poly rC)	0.002	0.01	72	18	62.5(*3*)	41.5(*11*)

* Concentration of polymer required to reduce formation of vesicular stomatitis virus plaques by 50 percent. Mean values for at least two observations. † From 3 to 24 hours after exposure of confluent cell monolayers in 60-mm petri dishes to 40 μg (preparation 1) or 8 μg (preparation 2) of the polymer in 1 ml of Eagle's minimal essential medium. Cells were freed from polymer after 3 hours, exhaustively washed with Eagle's medium, and further incubated with 4 ml of Eagle's medium per petri dish. Mean values for at least two observations. ‡ Not tested. § As observed in 0.01*M* tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 8.1, 0.01*M* Na_2HPO_4, 0.5 m*M* $MgCl_2$ (*7*); coincides with T_m value obtained in sodium citrate buffer (0.15*M* Na^+) (*9*).

acid • polyribouridylic acid [(poly rA) • (poly rU)] and polyriboinosinic acid • polyribocytidylic acid [(poly rI) • (poly rC)]. Interferon production and cellular resistance to bovine vesicular stomatitis virus (Indiana strain) was determined in human skin fibroblasts as described previously (*3*). The homopolymer pairs (poly rA) • (poly rU) and (poly rI) • (poly rC) were prepared by mixing equimolar amounts of the individual homopolymers (*6*). The alternating copolymer poly r(A-U) and its analog, poly r(AS-US), were prepared essentially by methods described (*7*). Both polymers showed a sharp thermal transition, suggesting a highly ordered, double- or multistranded secondary structure (*5*, *7*). Polymer concentrations were determined spectrophotometrically (*3*). Interferon production was also measured in the mouse, either in vivo after intraperitoneal injection, or in vitro in peritoneal macrophages (*8*).

A 100- to 10,000-fold lower concentration of poly r(AS-US) than of poly r(A-U) was required to reduce the formation of vesicular stomatitis virus plaques in human skin fibroblasts (Table 1). When both polymers were used at identical concentrations (40 or 8 μg/ml), poly r(AS-US) stimulated 2 to 20 times more interferon production than poly r(A-U); the difference in the stimulation of interferon production between the analog and the parent material is somewhat less striking than the difference in cellular resistance to the virus. This is most likely due to the fact that the interferon induction experiments were performed with concentrations of the polymer which gave invariably 100 percent virus plaque reduction. The homopolymer pair (poly rI) • (poly rC) and poly r(AS-US) did not differ significantly in induction of interferon, but poly r(AS-US) proved superior in inducing cellular resistance to virus infection. On the other hand, poly r(A-U) (preparation 1) was more effective than the homopolymer pair (poly rA) • poly rU). No interferon production could be detected with (poly rA) • (poly rU) under the given conditions. The polyribonucleotides poly r(AS-US), poly r(A-U) and (poly rI) • (poly rC) all had melting temperatures (T_m) greater than 60°C in 0.15*M* Na^+ and greater than 40°C in 0.01*M* Na^+, and were able to induce interferon production. The homopolymer (poly rA) • (poly rU) with a T_m under 60°C in 0.15*M* Na^+, and under 40°C in 0.01*M* Na^+, did not. Hence the 60°C (or 40°C in 0.01*M* Na^+) melting zone (*3*) seems important in predicting the interferon stimulating capacity of polyribonucleotides.

The poly r(AS-US) (preparation 1) stimulated about tenfold lower interferon amounts than (poly rI) • (poly rC), both in mouse peritoneal macrophages in vitro and in the serum of the whole animal. In representative experiments 15 and 140 unit/ml of interferon were detected after 8 hours in the supernatant fluid of macrophages exposed to 20 μg/ml of poly r(AS-US) or (poly rI) • (poly rC), respectively; 25 and 300 unit/ml of interferon were obtained in the serum of mice injected intraperitoneally with 10 μg per mouse of either poly r(AS-US) or (poly rI) • poly rC) 6 hours before. This dose of poly r(A-U) (10 μg, preparation 1) failed to stimulate detectable amounts of interferon (<10 units) in the intact animal. The antiviral activity, as in our human cell experiments, was characterized as interferon in that it was not active on cells of a different species and was destroyed by incubation with trypsin.

Whereas the greater antiviral activity of poly r(A-U) as compared to (poly rA)•(poly rU) could be related to differences in thermal stability, other factors were required to explain why poly r(AS-US) consistently surpassed poly r(A-U) in antiviral activity since poly r(AS-US) and poly r(A-U) had identical T_m values. To test whether poly r(A-U) was more readily degraded by nucleases, poly r(AS-US) and poly r(A-U) were exposed to various concentrations of pancreatic ribonuclease (Sigma, crystallized five times) for 1 hour at 37°C (*p*H 7.5), and their residual antiviral activity measured in human skin fibroblasts against vesicular stomatitis virus (Fig. 1). The poly r(AS-US) was 10 to 100 times less sensitive to ribonuclease than poly r(A-U). On the other hand, poly r(AS-US) protected human skin fibroblasts from subsequent virus infection at a 100-fold lower concentration than poly r(A-U). This parallelism does not necessarily reflect a

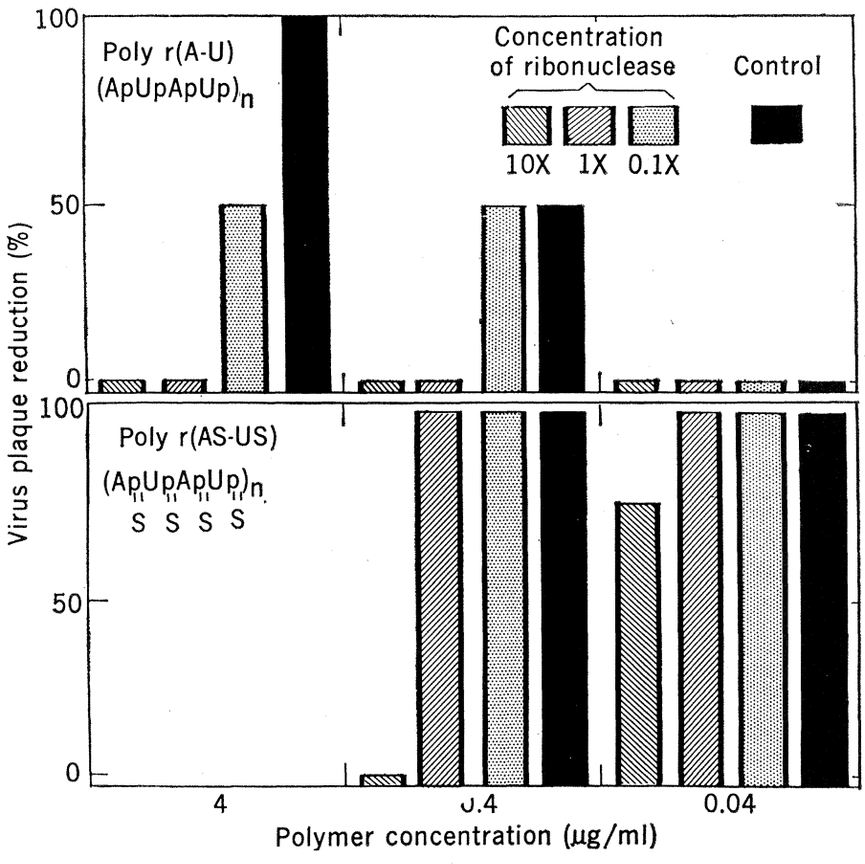

Fig. 1. Antiviral activity of poly r(AS-US) (preparation 1) and poly r(A-U) (preparation 1) after exposure to various concentrations of pancreatic ribonuclease. Polymers were exposed to increasing amounts of pancreatic ribonuclease for 1 hour at 37°C in the presence of $10^{-3}M$ ethylenediaminetetraacetate, in Eagle's minimal essential medium, *p*H 7.5. The ratio of enzyme to polymer concentration on a weight basis was 10, 1, and 0.1, respectively. Residual antiviral activity was measured in human skin fibroblasts against vesicular stomatitis virus.

causal relationship between ribonuclease susceptibility and antiviral activity. Both effects could be independent and result from important, and as yet undetermined, changes in three-dimensional structure. Colby and Chamberlin (*4*) followed the rate of ribonuclease degradation by measuring the increase of optical density at 260 nm of various ribonucleotide polymers and did not find any correlation between their efficiency of viral inhibition and sensitivity to pancreatic ribonuclease for the polynucleotides tested. However, the rate of breakdown of the polymers by intracellular nucleases may differ from their sensitivity to pancreatic ribonuclease. Nevertheless, it is tempting to causally relate the parallel increase of antiviral activity and resistance to ribonuclease degradation in poly r(AS-US).

As the substitution of a sulfur atom for an oxygen atom in the phosphate groups of poly r(A-U) results in a significant potentiation of the capacity to induce interferon production, it is likely that other, more active polynucleotides will also demonstrate greater activity upon sulfur substitution.

Note added in proof: Because of the excellent susceptibility of the rabbit to interferon inducers, poly r(A-U) and poly r(AS-US) were compared for their interferon inducing capacity in rabbits. Five to 15 and 600 to 1000 unit/ml of circulating interferon were detected 2 to 4 hours after intravenous injection of 20 μg of poly r(A-U), (preparation 2) and poly r(AS-US) (preparation 2), respectively. Thus, substitution of thiophosphate for phosphate in poly r(A-U) resulted in a more than 40-fold increase of interferon production in the rabbit.

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Cytogenetic Studies in Rats of Cyclohexylamine, a Metabolite of Cyclamate

Abstract. *Cyclohexylamine, the major known metabolite of cyclamate, was tested in vivo for possible cytogenetic effects. In rats injected with this metabolite, there was a direct relation between dose concentration and percentage of spermatogonial and bone marrow cells showing chromosomal breaks. Single chromatid breaks predominated with infrequent exchange figures.*

Consumption of cyclamate has now reached such a level that 13 million pounds were produced in 1967 and a production of 20 million pounds is projected for 1970 (*1*). At the time of its introduction, it was believed to be almost totally excreted and to have no metabolic products. Several investigators have since found that cyclamate is metabolized to cyclohexylamine (CHA) in dogs and man (*2*). The increase in the consumption of the cyclamates and the realization that metabolic products exist necessitates a reexamination of the entire subject of cyclamate safety.

The most difficult areas in which to determine a cause-and-effect relationship in the human population are in the assessment of carcinogenicity, mutagenicity, or teratogenicity after exposure of the subject to a specific compound. Because of the long latent period between exposure and expression of effects as well as the high background rate of damage, it is difficult to detect effects of a given agent in the population even after years of exposure. Induction of chromosome damage is the method we used to evaluate potential carcinogenic, mutagenic, or teratogenic effects of this cyclamate metabolite.

Holtzman strain albino male rats (170 to 200 g) received daily intraperitoneal injections for 5 days of 50, 40, 20, 10, or 1 mg/kg of CHA (*3*) (adjusted to *p*H 7.3 with HCl); controls received distilled water. For each dose at least 20 (usually 30) animals were used. The total amount of CHA given to the rats was 250, 200, 100, 50, or 5 mg/kg. The rats were killed 24 hours after the last injection of CHA. Cytogenetic studies were performed on both somatic and germinal cells.

Colcemid (*4*) (4 mg/kg) was administered intraperitoneally 5 to 5.5 hours before the tissue was taken. The bone marrow cells were collected from the femur by aspiration into Hanks' balanced salt solution (HBSS). The cells were then centrifuged at 1000 rev/min for 10 minutes, washed once again with HBSS, and exposed to hypotonic KCl solution (0.055*M*) for 30 minutes; they were then centrifuged and fixed in a mixture of methanol and acetic acid (3:1) for at least 30 minutes and usually for 24 hours. The fixative was changed once during this time; an air-dry preparation was then made on the slides. The slide preparations were stained by Giemsa (10 percent) with NH_4OH (5 ml of 0.15*N* NH_4OH per 100 ml of stain) for 5 minutes, rinsed in acetone, cleared with xylene, and mounted.