Uptake and Fate of Water-Soluble, Nondegradable Polymers with Antiviral Activity in Cells and Animals

Lalita Noronha-Blob, Vitolis E. Vengris, Paula M. Pitha, and Josef Pitha*

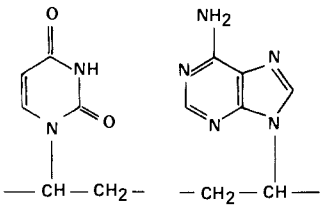
Laboratory of Molecular Aging, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224, and The Johns Hopkins University School of Medicine, Oncology Center, Baltimore, Maryland 21205. *Received September 17, 1976*

Poly(9-vinyladenine) and poly(1-vinyluracil) which are nondegradable, soluble polymers are taken up partially by mammalian cells grown in culture. The polymers remain associated with cells for several generations. In mice, after ip application, polymers slowly accumulate in liver, spleen, and thymus and remain there for as long as a month. Thus, these polymers which suppress the replication of murine leukemia viruses also accumulate in organs where the virus replicates. However, their antiviral activity does not reflect the amount of polymer found in these animal tissues. We propose that the polymers are gradually segregated into a group of cells or into subcellular organelles away from primary sites of virus replication. The results suggest that for a directly acting polymeric drug, a half-life over 24 h is without advantage.

Synthetic polymers have been used in medicine for the replacement of tissues,[1,2] as a substitute for human blood plasma,[3,4] and as carriers for different drugs in order to prolong the duration of their activity.[5,6] In addition, polymers can serve as chemical reagents[7-9] and as biologically active macromolecules with a capacity to inhibit the growth of tumors,[10-15] viruses,[16-19] and bacteria.[20-22] However, polymeric drugs vary in their reactivity and toxicity; they present problems of excretion[4,23] and are currently used only to a limited extent. The purpose of this investigation is to study some aspects related to the stability of macromolecules which would lead to the synthesis of effective compounds.

In this report the uptake and fate of two polymers, poly(9-vinyladenine) and poly(1-vinyluracil) [abbreviation: poly(vA), poly(9-vinyladenine); poly(vU), poly(1-vinyluracil)] (Chart I), were followed in mammalian cells in culture and in mice. Both polymers are electroneutral and stable to chemical and enzymatic hydrolysis. They form complexes with complementary polynucleotides and like polynucleotides they possess distinct antiviral properties against murine leukemia viruses.[16,18,19,24] Since they are not degraded in the body but accumulate in organs where virus replicates, we were able to investigate whether such indestructible polymeric drugs also persist in their antiviral activity. This question was of interest since the polymers are not toxic either to mice or to human cells in culture, as judged by cell number and the synthesis of DNA, RNA, and proteins.

Chart I



Experimental Section

Synthesis of Radioactive Polymers. Poly(vU), prepared by the procedure previously described,[16] was methylated by [^{14}C]methyl methanosulfonate (56 mCi/mol) in aqueous 0.1 N NaOH at room temperature. The methylation occurs in the N-3 position of the uracil residue; the product contained less than one methyl group per 100 uracil residues. Its UV maximum was at the same wavelength as that of the starting poly(vU) (263 nm) and after exhaustive dialysis it had 0.6 mCi/mmol of base residue.

Poly(vA) prepared as described previously[16] was labeled by two procedures. In the first procedure [^{3}H]ethyl methanosulfonate (70 mCi/mmol) was used to alkylate poly(vA) in the N-1 position of the adenine residue; the ethyl group was transferred to the N-6 position by subsequent treatment with aqueous alkali. The resulting product, after exhaustive dialysis, had a specific activity of 20 μCi/mmol of base residue and the wavelength of UV maximum was the same as that of the starting material (254 nm). The second procedure used for alkylation of poly(vA) involved the rapid freezing of a solution of poly(vA) (24 mg) and [^{14}C]-$ClCH_2COOH$ (4 mg, 100 μCi) in 4 ml of water to −20 °C. The mixture was left at the temperature overnight and then incubated at 37 °C for 2 days followed by extensive dialysis. The product had 2 μCi/mmol of base residue and its UV maximum was the same as that of the starting material.

Cells in Culture. Mouse fibroblast NIH 3T3 cells and human embryo lung cells, WI-38, were grown in modified McCoy's medium and minimal essential medium, respectively, both supplemented with 10% fetal bovine serum (Grand Island Biological Co., N.Y.).

The uptake of polyvinyl analogues into cells was measured by the addition of radioactive polymers into the growth medium of 2-day-old monolayers which were grown directly in scintillation vials (Packard). Following incubation at 37 °C in atmosphere of 5% CO_2, cells were washed extensively with ice-cold medium containing unlabeled polymer and solubilized in 1 ml of NCS (Amersham/Searle).

The fate of the polymer, poly(vA), in cells was investigated as follows. Cells which had been grown in plastic flasks (75 cm^2) overnight were exposed to radioactive polymer in a small volume of medium. After 3 h the cells were supplemented with additional medium and grown to achieve confluency (3–4 days). The monolayer was then rinsed with medium until no radioactivity was detected in washes and dispersed by treatment with 0.1% trypsin.[25,26] An aliquot of cells was transferred to fresh growth medium (generation 2) and allowed to grow until confluent. Samples of the medium and of the cells were monitored for radioactivity to determine the amount of polymer that was excreted and retained by the cells.

For isolation of subcellular fractions,[25] trypsinized cells were swollen for 20 min at room temperature in a hypotonic solution of sodium phosphate of pH 7.4 (1 mM) and disrupted in a Dounce homogenizer. The suspension was then made 0.25 M in sucrose and fractionated by centrifugation. Nuclei and cell membranes were sedimented at 500g for 10 min. A lysosomal–mitochondrial fraction was collected following centrifugation at 12 500g for 20 min and a microsmal fraction was sedimented at 105 000g for 30 min. The radioactivity associated with each fraction was measured in Aquasol (New England Nuclear, Boston, Mass.). Aliquots of the fractions were then treated with Triton X-100 (final concentration 1%) for 10 min and centrifuged as indicated before to estimate the amount of polymer released from the membrane encapsulated form.

Animal Study. DBA/2 mice were housed in metabolic cages and injected ip with radioactive polymer: 1 mg per mouse of

* Address correspondence to this author at the National Institute on Aging.

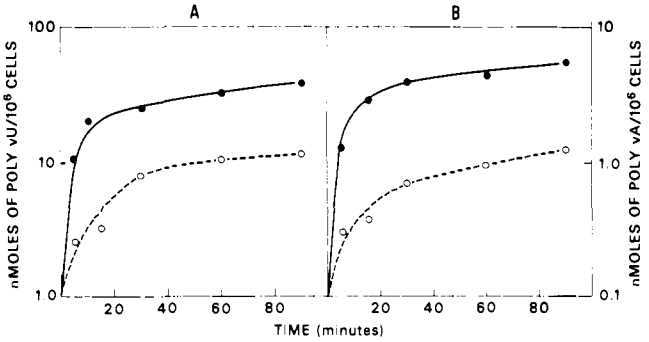


Figure 1. The uptake of (A) poly(vU) and (B) poly(vA) by 3T3 cells. Uptake from 10^{-3} M solution (●—●), from 10^{-4} M solution (○---○); concentrations of polymers are expressed on monomer basis.

[^{14}C]poly(vA) and 0.3 mg per mouse of [^{14}C]poly(vU). Urine and stool were collected every day for the measurement of excreted radioactivity. At each of several times postinjection one mouse was killed by cervical dislocation; tissues were quickly removed, blotted, weighed, and either immediately oxidized or stored at −20 °C until further processing. Packard Model 305 Tri-Carb sample oxidizer was used.

Polyacrylamide Gel Electrophoresis. The molecular weight distribution of poly(vA) found in liver, spleen, and urine was estimated by polyacrylamide gel electrophoresis according to the procedure described.[27] Mice injected ip with [^{14}C]poly(vA) were sacrificed after 3 weeks to obtain liver and spleen samples; urine samples were collected at 2-day intervals. Homogenates of each sample were prepared in a Dounce homogenizer, carrier cold poly(vA) was added, and they were made to a final concentration of 1% SDS and 1% 2-mercaptoethanol. After heating at 100 °C for 1 min, they were subjected to electrophoresis on 10% acrylamide–SDS gels previously described,[27] which were prerun at 60 V for 30 min. Electrophoresis was performed at 60 V for 3 h after which the gels were sliced, incubated overnight in NCS, and mixed with 10 ml of scintillation fluid.

Results

Polymers and Cells in Culture. The kinetics of uptake of poly(vA) and poly(vU) into 3T3 cells is shown in Figure 1. The uptake, which is dependent upon the concentration of polymer, slows down after 30–60 min. The final amounts of poly(vU) taken up by the cells is about ten times greater than that of poly(vA) and corresponds to about 0.4 and 0.04%, respectively, of the total polymer used. Approximately 0.1–1 μg of polymer remains firmly associated with 10^6 cells; this represents about 0.02% of the dry weight of cells.

To determine whether polymer is retained by cells through several generations or is excreted into the growth medium, mouse and human cells were first grown in the presence of [^{14}C]poly(vA) for 48–72 h, at concentrations that are not toxic to the cells. (ref 16 and unpublished observation). At confluency, monolayers were dispersed and subcultured. Table I shows that the amount of radioactive polymer found in the confluent monolayers of generations 2 and 3 approximately corresponds to the number of viable cells that had attached; thus poly(vA) remains in the cells from one generation to the next. No significant quantities of polymer are eliminated in the medium due to cellular metabolic processes or to the preferential death of the polymer-containing cells.

The retention of poly(vA) by cells following trypsinization indicates that the polymer is located intracellularly rather than at the cell surface. Fractionation of cells containing polymer into subcellular components also led to the same conclusion. Table II shows that in the case of 3T3 about half the amount of polymer is associated with the lysosomal–mitochondrial fraction. Most of this remains bound and is only partly released from the membrane encapsulated form by Triton (not shown); the other half remains in the soluble fraction of the cytoplasm. In the case of WI-38, about one-third of poly(vA) is found associated with each cell fraction. In the control experiment unlabeled WI-38 cells were fractionated in the presence of radioactive poly(vA). As shown in the last column of Table II very little polymer is found in fractions containing the nucleus, lysosomes–mitochondria, and ribosomes; similar results were obtained using 3T3 as control.

Table I. Fate of Polymer in Cells in Culture: Cell Associated [^{14}C]Poly(vA)

Generation[a]		Cell line, cpm WI-38	Cell line, cpm 3T3
1	Confluent monolayer after polymer absorption	1825	1786
2	Cell inoculum[b]	912	846
	Attached cells	675[c]	245[d]
	Confluent monolayer	570	252
3	Cell inoculum	285	119
	Attached cells	211	36
	Confluent monolayer	160	26

[a] One generation refers to more than one doubling of cells (compare data on cell attachment); for the first generation WI-38 cells of passage 23 were used. [b] Half of the cells from each generation was used as inoculum for the subsequent one (see Methods). [c,d] Calculated on the basis of plating efficiency: 74% of the WI-38 cell inoculum and 30% of the 3T3 inoculum attaches in each generation/passage (ref 26 and unpublished data).

Table II. Distribution of [^{14}C]Poly(vA) in Cells in Culture (Percent of Total Radioactivity Associated with Cells)

Cell fraction	Cell line 3T3	WI-38[a]		Control[a,b]	
Nuclei, cell membranes and unbroken cells	6	26	35	2	3
Lysosomes, mitochondria and microbodies	43	19	33	1	2
Ribosomes and fragments of endoplasmic reticulum	3	5	19	4	5
Soluble fraction of cytoplasm	50	18	19	62	52

[a] Two representative experiments are presented. [b] Radioactive poly(vA) was added to unlabeled disrupted WI-38 cells.

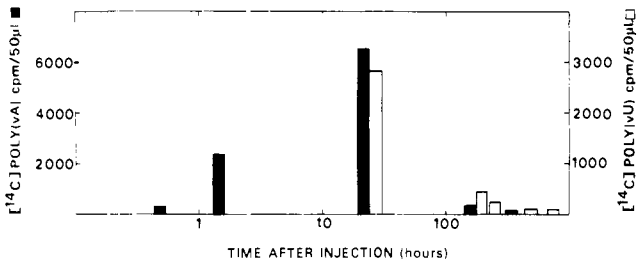


Figure 2. The amount of poly(vA) (■) or poly(vU) (□) in circulating blood of mouse at various times after ip injection.

Fate of Polymers in Animals. To study the effects of polymers in vivo, radioactive polymer was injected into the peritoneum of mice in doses which did not produce toxicity.[24] Figure 2 shows that polymers quickly appeared in the blood stream and although their levels decreased rapidly after 24 h, trace amounts were detected even after several weeks. Considerable quantities of the administered

Table III. Distribution of Vinyl Polymers in Tissues of Mice at Various Times (Days) after ip Administration[a]

Tissue	[^{14}C]Poly(vA), cpm/mg								[^{14}C]Poly(vU), cpm/mg				
	Experiment 1				Experiment 2								
	7[b]	10[b]	20[b]	30[b]	0.25[b]	5[b]	7[b]	21[b]	0.25[b]	1[b]	3[b]	7[b]	14[b]
Liver	4	7	25	29	36		316	355	150	132	154	174	253
Spleen	9	15	18	28	7	408	423		73	66	72	48	190
Thymus		17		30									
Heart	10	6	7	4	14	8	14		4	3	4	5	5
Lung	6	7	5	3	2		11	27	10	13	28	22	13
Gastrointestinal tract	3	0.5	0.5	0.7	15	6	10		19	9	19		14
Kidney	7	5	0.2	0.2	29				69	22	36	30	26
Brain	0.3	0.5	0.1	0.1					0.3	0.4	0.5		0.7

[a] Protocol described in the Experimental Section; the specific activity of poly(vA) used in the second experiment was 20 times higher than that of experiment 1; poly(vU) data are representative values of two experiments. Background counts per minute were between 0.12 and 0.34 cpm/mg. [b] Days.

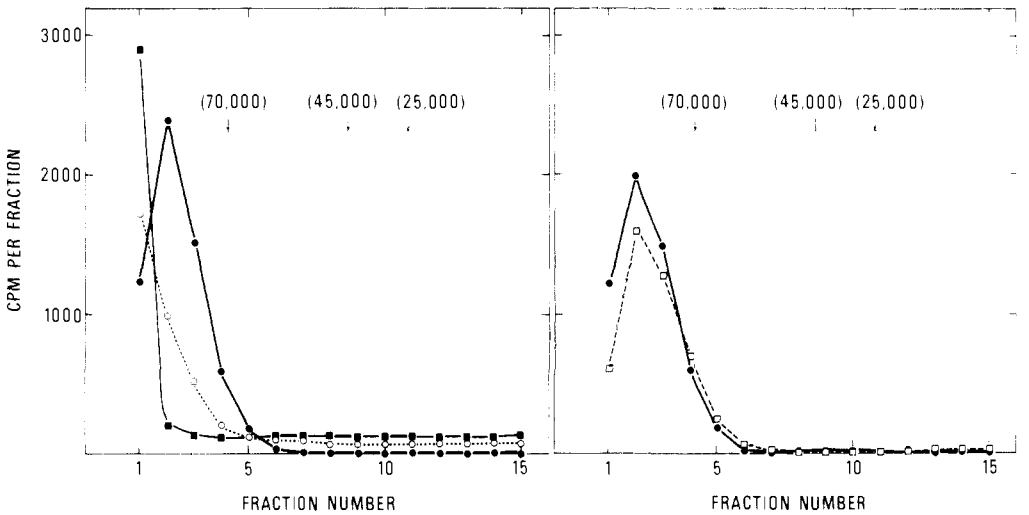


Figure 3. Polyacrylamide gel electrophoresis of poly(vA) in homogenates of liver (■—■) and spleen (○---○) 3 weeks postinjection, in urine excreted 3 days postinjection (□—□), and in the polymer used for injection (●—●). Protein standards (bovine serum albumin, chymotrysin, and ovalbumin) were electrophoresed under identical conditions.

dose are lost from the body via stool and urine during the first 24 h: 20% of poly(vA) and 22% of poly(vU).

The distribution of the injected polymers in various tissues was next studied. Altogether four experiments were performed in which different protocols were followed. The conclusions were the same; representative results are presented in Table III. The amount of poly(vA) found in liver, spleen, and thymus increases with time and is relatively higher than that found in other tissues after 30 days. Eventually about ten times as much polymer accumulates in liver and spleen, compared to other tissues. Interestingly, while the amounts of both polymers remain nearly constant in heart and lung, it appears that the kidney and the gastrointestinal tract are partly cleared of the polymers. Very little radioactivity was detected in brain tissue at all times.

The long-term retention of polymers in animal tissues suggests that these compounds are not metabolized. The same conclusion can also be drawn from results of the following experiments. When urine containing the excreted poly(vA) was dialyzed, 97% of the radioactivity was retained indicating that poly(vA) is not degraded in the body to low-molecular-weight material. Furthermore, as shown by SDS–polyacrylamide gel electrophoresis in Figure 3, the molecular weight distribution of the poly(vA) excreted into the urine and that accumulated in the spleen and liver, even after 3 weeks, is comparable to the starting polymer which was administered. These experiments were carried out at the suggestion of a reviewer. The gel concentration used was chosen to detect any small molecular weight polymer if it were present; this did not permit complete entry of the very large molecular weight material into the gel.

Discussion

Both polymers enter cells grown in culture. The final amounts taken up (about 0.1–1 μg per 10^6 cells) are comparable to the amount of foreign RNA or DNA which enters cells[28] but are higher than that of foreign polysaccharide.[29] However, the rate of uptake of these polymers is about the same as that of polysaccharides and is much slower than that of polynucleotides.[30] The rate of uptake suggests the possibility of pinocytotic entry, which is believed to be of universal occurrence in cells in culture.[31,32] However, considerable amounts of polymer are not encapsulated in vesicles but are found in the soluble fraction of the cytoplasm.

In animals, foreign macromolecules are known to enter cells, predominantly of the reticuloendothelial system, by two mechanisms—pinocytosis and phagocytosis.[31] In mice, the polymer enters the blood stream within 1 h and remains there in appreciable concentrations up to 24 h. About one-third of poly(vA) is excreted by the kidney. The rest is retained in tissues, particularly in the spleen and liver, which suggests that organ-fixed macrophages are active in this process.[31] The excreted poly(vA) is apparently not degraded in the body; such nondialyzable polymers have been previously detected in urine.[3,4,13,14]

Both poly(vA) and poly(vU) suppress the replication of murine leukemia virus (MLV) in cells in culture[16] and in

mice without involving either a cell mediated or humoral immune response.[24] It was found that in order to inhibit MLV in mice, small doses of polymer had to be administered daily; a cumulative high dose injected once was ineffective.[24] Thus, circulating polymer was required for antiviral activity to be expressed. This observation coupled with those reported here provides some information concerning the active form/site of these compounds. We have shown that poly(vA) accumulates in the spleen of mice, an organ where murine leukemia virus replicates. Furthermore, by the parameters examined (electric charge and molecular weight distribution), this accumulated polymer is very similar to the administered control even after 3 weeks. Thus, the polymer contained in mouse organs should sustain its antiviral activity. However, as mentioned above, daily administration of polymer was required for virus inhibition. These results show that the accumulation of polymer in tissues is accompanied by its gradual conversion to an inactive form. This conversion may be explained by the hypothesis that the polymer is segregated into certain cells within the tissue or into compartments within the cell, away from primary sites of viral replication.

Conclusion

Judged by the rate of uptake of polymers into cells in culture and their relatively rapid distribution to different tissues in mice, it appears that the macromolecular nature of drugs does not slow down their action drastically. However, to obtain optimal pharmacological activity with electroneutral polymers, compounds with a short half-life (greater than 1 h but not over 1 day) should be synthetized. Nondegradable polymers, even when nontoxic, are without any notable advantage as they lose their biological potency in animals probably by being segregated into different cells or into subcellular components where they cannot influence virus replication.

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Disteroidyl Ethers. 1. Synthesis and Oral Long-Lasting Uterotrophic Activity of 1,3,5(10)-Estratrien-17-yl Enol Ethers of 3-Keto Steroids

Romano Vitali, Serafino Gladiali, Giovanni Falconi, Giuseppe Celasco, Maria Adele Saccani, and Rinaldo Gardi*

Vister Research Laboratories, Casatenovo (Como), Italy. *Received January 7, 1976*

A series of disteroidyl enol ethers derived from a 17-hydroxyestratriene and a 3-keto steroid has been synthetized and tested for prolonged uterotrophic activity after oral treatment. Most of the compounds derived from 17β-estradiol displayed a high, prolonged activity, many of them being more active than quinestrol.

In the last 15 years many steroid ethers have been investigated in our laboratories and several of them proved to be outstanding for their oral activity. In particular, compounds like 3-cyclopentyl ethers of ethinylestradiol and other estrogens,[1] as well as estradiol 17-cycloalkenyl ethers,[2] displayed a quite unusual oral long-lasting activity.