Vinyl polymer analogues of nucleic acids*

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The preparation and properties of vinyl polymers carrying the bases of nucleic acids as substituents are described. These compounds form specific complexes with complementary polynucleotides, a process which prevents the latter from functioning as templates in the synthesis of polymeric nucleic acid or protein. The uptake and fate of these polymers in mammalian cells grown in culture and in animals are described; these polymers exhibit weak but selective antiviral effects in such systems.

The biosphere is rich in polymers – the contents of living cells are more or less solutions of natural polymers in aqueous electrolyte, and man-made polymers represent an integral part of our environment. The vinyl polymeric analogues of nucleic acids described herein have structural elements of both of these groups of macromolecules. They have, as substituents on a polymeric chain, the heterocyclic moieties of nucleic acids, and these are attached to a backbone which is the same as in common plastics – a backbone formed by the polymerization of a vinyl compound. The structure of these analogues is shown in *Figure 1*; also illustrated are the main differences between nucleic acids and the present analogues. Analogues have shorter distances between substituents, do not carry electric charges and cannot be broken down by chemical or by enzymatic hydrolysis.

These polymers have been prepared only relatively recently, by a free radical polymerization of the corresponding *N*-vinyl monomers. The necessary monomers were prepared by the elimination of hydrogen chloride from the corresponding β-chloroethyl derivatives[1–4] or by pyrolysis of the β-acetoxyethyl derivatives[1]. The starting compounds for these reactions can easily be made from commercially available chemicals. An alternative method of preparation uses a vinyl exchange reaction between vinyl acetate and the parent heterocyclic compound[1,5–7]; a procedure which simplifies the isolation of the reaction product has been recently described[8]. Similar methods were used for the preparation of related monomers[9–16].

Poly(9-vinyladenine) (polyVA in *Figure 1*) prepared by direct radical polymerization, is quite water-soluble (up to 10 mg/ml)[3,17,18]. Alternative preparation of polyVA, from poly(6-chloro-9-vinylpurine) by reaction with ammonia, yields a compound which is less soluble in water, probably due to some crosslinking during the substitution reaction[19]. PolyVA can be fractionated on the basis of its molecular weight by the gel filtration[16,20,21] or gel electrophoresis method in the presence of sodium dodecyl sulphate[20]. The latter fractionation is based on the ability of anionic detergent to bind to the electroneutral polymer by intercalation; polymer thus acquires an electric charge and a rigid conformation. The physical and hydrodynamic properties of polyVA were studied in detail[19,21,22].

The polymerization of 1-vinyluracil yields a polymer (polyVU in *Figure 1*) which is only sparingly soluble in water and probably crosslinked[2,23,24]; when a protected monomer, e.g. 1-vinyl-4-ethoxy-2-pyrimidinone, is polymerized and the protective groups are then removed by mild hydrolysis, a polyVU with higher water solubility (up to 10 mg/ml) results[19,23]. Poly(1-vinylcytosine), (polyVC) was prepared by the reaction of the above mentioned protected polymer with ammonia[25]. The water solubility of the different preparations of this polymer vary, indicating that in the reaction with ammonia some crosslinking occurs; nevertheless the majority of such preparations are well-soluble in water–glycol mixtures[25]. Poly(9-vinylhypoxanthine)(polyVI) which is an analogue of poly(inosinic acid), is soluble only in mixtures of solvents or in a solution of an anionic detergent, which is again intercalated into polymer[17]. In addition to these compounds, other similar polymers were prepared, e.g. a water-soluble poly(9-vinylpurine)[26], also poly(7-vinyltheophylline)[27] and poly(3-methyl-1-vinyluracil)[19,23] which are water-insoluble, and poly(7-vinylpurine), which was found to lose water solubility with

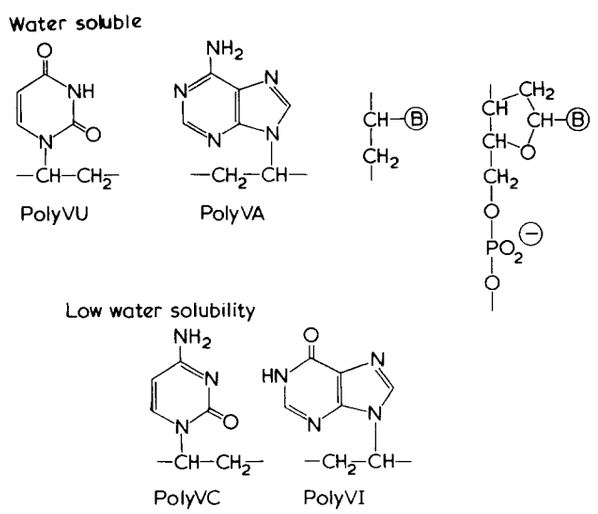

Figure 1 Structures of vinyl analogues of nucleic acids. On the right, the back-bones of vinyl polymer and deoxyribonucleic acid are compared; B denotes a base

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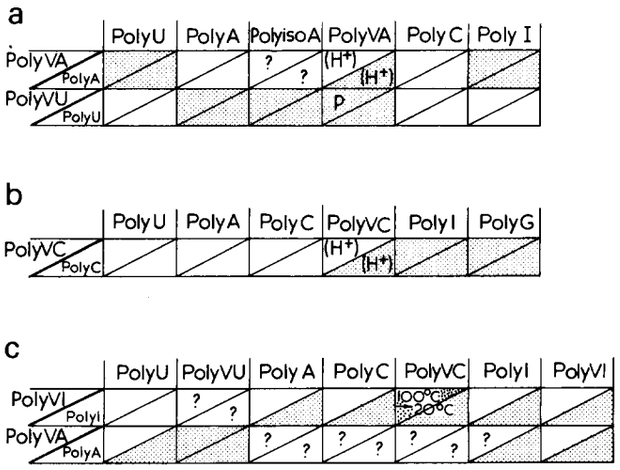

Figure 2 Formation of complexes between the vinyl analogues and polynucleotides as detected by spectral changes. Shaded areas, complex; unshaded areas, ⊖ complex; p, precipitate. (a) Aqueous solutions; (b) water–propylene glycol solutions (c) aqueous SDS solutions

time, or after drying. Furthermore, several copolymers were also prepared[4,28–31].

Heterocyclic substitutents in nucleic acids form pairs through hydrogen bonds with their complementary partners (uracil with adenine, cytosine with inosine); double stranded structures are thus achieved. The present analogues can form similar complexes[17,18,23,32]; the results of a study of all possible combinations by u.v. spectroscopy[17] are summarized in *Figure 2*. Complex formation in *Figure 2* is denoted by the dark colour; the lower right part of the Figure indicates whether or not there is a complex formed between the particular combination of polynucleotides; the upper left part indicates whether or not there is complex formation between the corresponding polynucleotide–analogue combination. The coincidence of colours in nearly all the fields in *Figure 2* shows that the specificity of complex formation in the natural and analogue systems are the same and that the specificity of the nucleic acids was transferred to the analogue system.

There are two main features which help in the understanding of all these complexes. The first involves the steric peculiarities of analogues; they are atactic vinyl polymers and there is, thus, no way in which strands of analogues can be arranged that leads to complete base-pairing with any other polymer; in all possible arrangements some bases are left free. In fact this particular situation was visualized by electron microscopy[17]. *Figure 3a* shows several molecules of the complex between the vinyl polymer and polynucleotide. On these ribbon-like structures knots can be seen in which the difference in the geometries of both backbones are expressed; the strand of analogue simply bulges out where a number of bases are incapable of forming base pairs. *Figure 3b* is an electron micrograph of double-stranded nucleic acid and no bulges or knots can be seen; the strands fit each other neatly with all bases fully bound. The incomplete base-pairing schemes naturally decrease the stability of the corresponding complexes. The second important feature in understanding complexes of analogues involves the electroneutral character of analogues. Nucleic acids are polyanions and there is thus considerable repulsion between any two strands. Since such repulsion is lacking in complexes of electroneutral analogues the strength of the complexes formed by these analogues tends to be increased.

Complexes between polynucleotides and analogues are formed in a specific way, and as these complexes are polyanionic they do not aggregate and remain as individual molecules; this situation has already been illustrated in *Figures 2* and *3*. On the other hand, a complex between two complementary analogues is electroneutral and the individual molecules aggregate to form a precipitate[19]; this situation is illustrated in molecular terms in *Figure 4*. Introduction of an electronegative charge into the analogue molecule prevents it from complexing with the complementary poly-

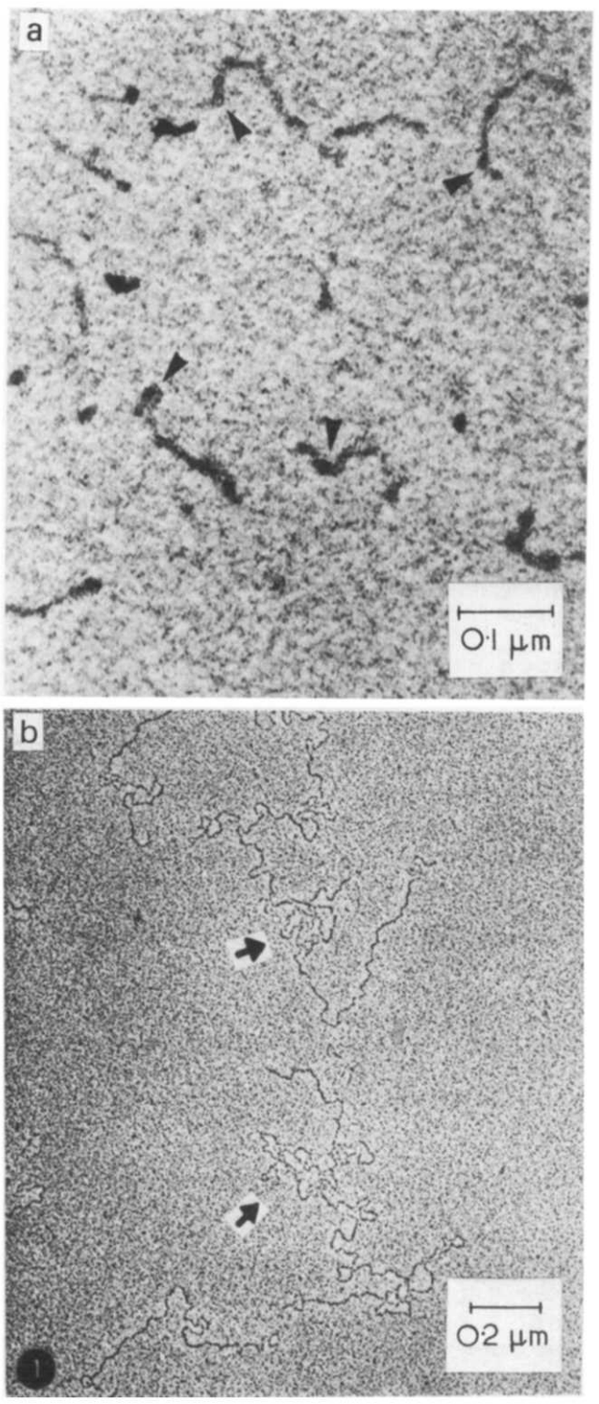

Figure 3 Electron micrographs (a) complex of polyVU.polyA; (b) double-stranded DNA

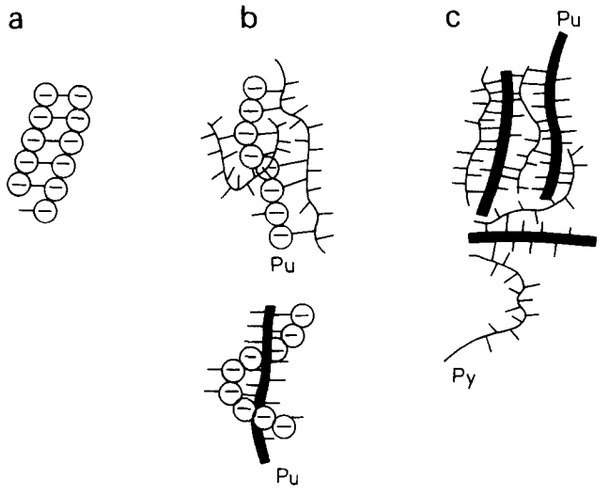

Figure 4 Scheme illustrating the formation of complexes between polynucleotides and analogues. (a) The polynucleotide–polynucleotide combination. (b) The structures of polynucleotide–analogue complexes; the stoichiometry of the complexes depends on whether or not the stiffer purine component is a polynucleotide or an analogue component; in all cases a number of bases are not paired. (c) Aggregation observed with complexes of the complementary vinyl analogues are illustrated

nucleotide. This introduction of charge can be achieved by an intercalation of electronegative detergent into the analogue: polyVA or polyVI, in the presence of sodium dodecyl sulphate, do not form any complexes with the complementary polynucleotides[17]. Electronegative charge can also be introduced into an analogue in a permanent manner – by copolymerization of the monomer with maleic anhydride; the resulting copolymers do not form any complexes with polynucleotides[30,31]. Among polynucleotides, poly(cytidylic acid) and poly(adenylic acid) form ordered structures in an acidic medium; but there is no similar complex formation with self by the corresponding analogues[19,25] (*Figure 2*). This can be easily explained: in polyanionic polynucleotides, the protonation decreases the total electric charge and thus favours association while in electro-neutral analogues it creates electric charge and this, together with its atactic structure, eliminates any tendency for self-association.

Adenosine by itself is slightly self-associated in concentrated aqueous solutions – the aromatic rings tend to stack on each other[33]. The transition in the polyVA conformation which was observed upon cooling of an aqueous solution of this polymer may be caused by such costacking of adenine residues; this transition can be easily detected by hydrodynamic methods[21] but causes only minimal changes in the ultra-violet spectrum of the polymer[16,21]. PolyVA shows a tendency to associate by costacking not only with itself, but also with poly(adenylic acid). As with the self-association, no interaction between poly(adenylic acid) and polyVA could be detected by spectral changes[17] but some binding was detected during the electrophoresis experiments[34]. Interactions of this type are probably very weak, and special methods are needed for detection of the few costacked bases.

PolyVU complexes not only with poly(adenylic acid) but (at low temperature) even with the monomeric units adenosine and adenylic acid[23,35]. This phenomenon was put to use in template synthesis, where adenosine cyclic 2′,3′-phosphate bound to the polyVU was polymerized by the addition of ethylenediamine[35]. The yield of this polymerization reaction was comparable to the one obtained in a reaction which had a natural polynucleotide as template.

The binding properties of polymeric analogues may find direct application in biochemistry. Polynucleotides immobilized on Sephadex or Sepharose are made commercially; their replacement by vinyl analogues would eliminate problems due to the sensitivity of these commercial preparations to hydrolysis. Vinyl analogues can be very simply immobilized by including them in polymerization mixtures which are based on the linkage of acrylamide units[34]. Gels thus prepared were used directly for electrophoretic separation of polynucleotides[34]; the stability of attachment is satisfactory even for affinity column chromatography, after mincing the gel to form beads.

Interactions of these analogues with proteins are probably weak. PolyVA immobilized on Sepharose binds an enzyme of the nucleic acid system, the viral DNA polymerase, but this binding is easily reversed by a protein which is not at all related to the nucleic acid system; bovine serum albumin[36]. Thus, binding is probably not only rather weak but non-specific as well. Furthermore, in all the investigations of cell-free systems described in this text, the binding of analogues to proteins was not found to be important. Analogues would be expected to intercalate lipoid compounds, e.g. phospholipids in a manner similar to the described intercalation of sodium dodecyl sulphate[20] but no experiments in that direction have been carried out.

Several other interesting interactions of the present analogues were observed. PolyVA and poly(9-vinylpurine) bind strongly to Sephadex[26], a modified polysaccharide. The substitution of the amino groups on polyVA by methyl groups lead to a large decrease in the binding of this polymer to Sephadex[26]; no binding was detected for polyVU.

Polymeric analogues exert specific effects both on enzymatic and on more complex cell free systems. Those effects of polyVA on enzymes which copy the deoxyribonucleic acid in mouse cells were studied in detail[27]. There are three classes of DNA polymerases in this case; if polyVA complexes with the polynucleotide which is being used by the polymerase as a template, any of the three enzymes are inhibited. The inhibition occurs even when the enzyme has already begun transcribing its template; the inhibited enzyme, apparently entangled in the analogue–polynucleotide complex, is unable to get free to use another template[27]. The action of analogues on the enzyme which transcribes deoxyribonucleic acid into ribonucleic acid was studied using polymerase isolated from *E. coli*[37]. A pattern similar to the above was found; in all cases where complexing between the analogue and template occurs, the enzymatic activity is blocked. There is another class of nucleic acid polymerases, enzymes which copy ribonucleic acid into deoxyribonucleic acid. This process, called reverse transcription, is essential to the life cycle of the leukemia viruses. The pattern of these analogues' effects on reverse transcription is complicated[36,38]; as above, the enzyme is inhibited when the analogue forms a complementary complex with the template. In addition polyVA influences even the reaction in which poly(adenylic acid) serves as a template. However, this reaction requires a primer, oligodeoxythymidylic acid, and polyVA may act through interaction with the template–primer complex; an interaction based on a weak interaction between polyVA and poly(adenylic acid) and on a strong interaction between polyVA and poly(deoxythymidylic acid). Another finding suggests that weak interactions may be important in the inhibition of this enzyme. Poly(9-vinylpurine) cannot form ordinary base pairs with any polynucleotide; nevertheless it is a good inhibitor of the reverse

transcriptase[26]. This inhibition is quite sensitive to steric hindrances; introduction of a dimethylamino group into position 6 of the purine ring completely eliminates any inhibitory effects[26].

The effects of these analogues on protein synthesis in cell-free systems were also investigated. When a polynucleotide is used as a messenger, the analogue which forms a complementary complex with that polynucleotide fully inhibits protein synthesis[29]. An interesting situation was observed using ribonucleic acid coding for globin[39]. This nucleic acid contains a sequence which is practically pure poly(adenylic acid). Its translation into protein was not influenced by polyVA; on the other hand, the reverse transcription of this compound into deoxyribonucleic acid, a reaction which requires oligodeoxythymidylic acid as a primer, was inhibited by polyVA[39]. Thus the necessity for a primer may significantly influence the effect of the analogue. It is interesting to note that when electronegative charges were introduced into polyVA, by the above mentioned copolymerization of the monomer with maleic anhydride, the pattern of effects on protein synthesis was completely changed[30,31]. Apparently, as soon as an analogue starts to mimic a nucleic acid by its polyanionic character, the relatively simple inhibitory effects, described above, are lost.

Human or animal cells grown *in vitro* represent a suitably simple system in which the biological effects of polymers can be studied. Macromolecules may become attached to the cellular membrane and thus influence cellular processes. Diffusion of a polymer through the cell membrane is an extremely slow process; but a polymer can enter the cell by pinocytosis (the process of 'cell drinking') or by penetration following damage to a portion of the membrane, a cellular apparatus which is capable of considerable self-healing. When human or mouse cells were exposed to a solution of polyVA or polyVU, a small portion of the polymer became firmly associated with the cells; about 0.1–1 pg per cell[40]. This amount is about in the middle of the range of macromolecular uptake; as much as 10 pg of protein (ferritin) may become associated with the cell[41] or as little as 0.01 pg of polysaccharide (dextran)[42]. Polymer which becomes associated with a cell is bound very firmly; when the cell divides nearly all of this polymer is passed on to the daughter cells, then to granddaughters and so on[40]. This is an important property; apparently soluble, non-degradable polymers can represent as much of a menace to living cells as non-degradable plastics do to our environment.

Cells, after disruption, can be fractionated into nuclear and cytoplasmic components. When cells containing vinyl polymers were disrupted, a considerable portion of the polymer was found in the cytoplasm, some of it free, some of it encapsulated in lysosomes(organelles which the cell uses in digesting proteins). Thus, the present analogues seem to enter, at least to some degree, the interior of cells[40].

Analogues show quite interesting toxicity effects. PolyVA is non-toxic to cells, while polyVU is toxic at high concentration (millimolar range) and then only to cells which are rapidly growing but not to quiescent cells[38].

In cell cultures the effect of polymers on the replication of viruses can also be tested[38]. PolyVA proved to be very interesting in this respect; it inhibits the replication of murine leukaemia virus. Inhibition is virus-specific, since replication of other studied viruses, Sindbis or vesicular stomatitis virus, was not inhibited[38]. PolyVA does not prevent the entry of the virus into cells; the inhibition probably concerns some early process of viral replication, presumably reverse transcription. The late processes of viral replication are not inhibited by the polymer[43].

It is interesting to note that another polymer which inhibits reverse transcriptase, poly(9-vinylpurine), also inhibits the replication of murine leukaemia virus[26]. On the other hand, if the amino groups in polyVA are replaced by methyl groups, the resulting polymer [poly(9-vinyl-6-dimethylaminopurine], neither inhibits reverse transcriptase nor inhibits the replication of leukaemia virus[26].

Several polynucleotides are known to induce a specific antiviral defense, the interferon system, in cells which are grown in culture. The present analogues themselves are unable to induce interferon. They have neither polyanionic nor stereoregular structure, conditions which are thought to be prerequisites for that induction. Thus, it came as a surprise when the complex between poly(inosinic acid) and polyVC was found to be a good interferon inducer[44]. The structural aspects of the induction phenomenon are shown in *Figure 5*. PolyI.polyC, a double stranded polynucleotide, is a potent inducer. Structural changes which are located inside of this molecule (*N*-acetylation in *Figure 5*) do not destroy the inducing ability of the complex, but as soon as the periphery of the molecule is changed (e.g. bulge formation by mispairing or by an occasional $2'-5'$ bond in *Figure 5*) the ability to induce interferon is completely lost. The complex between poly(inosinic acid) and polyVC is not stereoregular and is, as mentioned above, aggregated in aqueous solution (*Figure 5*). Despite this, the complex is a good interferon inducer, indicating that other factors must apparently be involved. At least one of these factors has been traced to the aggregated character of the compound; aggregation leads to a very high efficient uptake of the com-

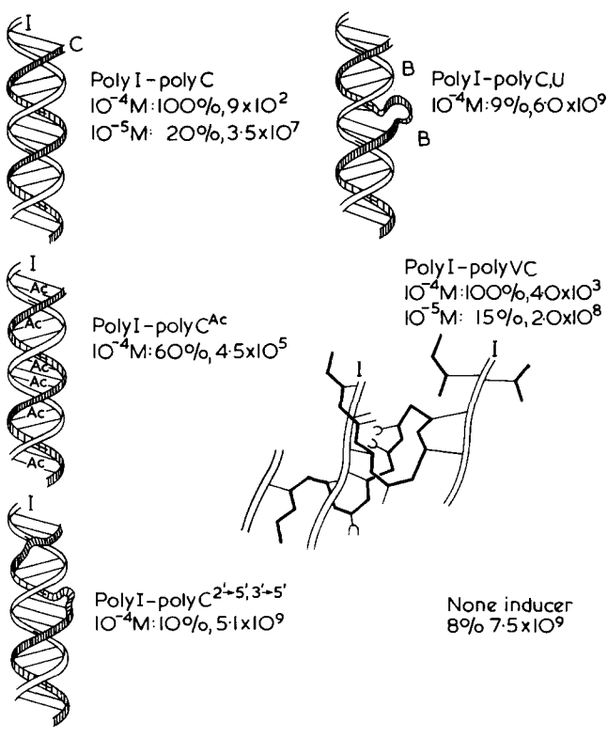

Figure 5 Scheme illustrating changes in the interferon inducing ability occurring upon changes in the structure of a polynucleotide inducer, polyI.polyC; any deformation of the backbone seems to cancel the antiviral activity. Below right is the structure of an analogue complex which is active in induction the activity is achieved by another mechanism. Numbers given refer to the infections by vesicular stomatitis virus

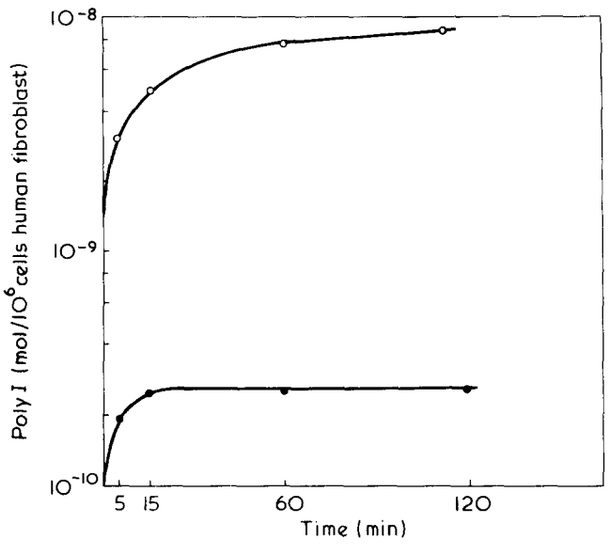

Figure 6 Uptake of polyI.polyC and polyI.polyVC by human cells grown in culture. ○, PolyIVC; ●, polyIC

plex by cells (*Figure 6*) and once inside a cell the steric requirements of the inducer are apparently much lower[44]. The entry of the complex into cells must be due to stimulation of pinocytosis or to partial membrane damage, since exposure of cells to this complex also increased the uptake of non-related proteins by these cells by two orders of magnitude[45]. Thus, a curious parallel has been achieved; the present analogues have many of the structural elements of plastic, and in this particular situation the analogues functioned much like their more worldly relatives, as molecular packing material, making shipping across the cellular membrane more effective.

The effects of polyVA on animals were also studied[40,46]. The compound is notably non-toxic and doses up to 1000 mg/kg are tolerated by mice. When polymer is injected into the peritoneum, within hours it is found in the blood stream in considerable concentration, which again gradually decreases after about a day. About a third of the polymer is excreted in the urine; the rest is retained in the body and slowly accumulates in liver, spleen and thymus and remains there for as long as a month. These organs contain a considerable number of cells of the reticuloendothelial system (e.g. Kupfer cells in liver) which are known to be involved heavily in pinocytosis (cell drinking) and phagocytosis (cell eating). These cells are probably the final location of these polymeric analogues in the body. The polymers found in urine, spleen and liver are non-dialysable. The amount of the small molecular weight fraction in these polymers was measured by gel electrophoresis and found to be comparable to that in the starting material[40].

Effects of polyVA in the mouse are rather selective. This non-toxic polymer does not influence the host's immune responses (cellular or humoral). Also, no adverse reactions due to antigenicity of the polymer were observed in the mouse; special conditions are usually necessary to make any soluble vinyl polymer antigenic[47]. In mice, polyVA administered daily inhibits the replication of Friend leukaemia virus, but does not influence the replication of another non-leukaemic virus (Semliki forest virus)[46]; the situation is thus, rather similar to that observed in cells grown *in vitro*. Furthermore, in the mouse polyVA does not induce interferon by other agents, nor inhibits the growth of L1210 tumour, which transplantable tumour is of non-viral origin. Thus, the effects of this polymer in the mouse seem to be directed specifically against leukaemia virus[46].

This inhibition is interesting from a mechanistic point of view, since polyVA accumulates in a non-degraded form in the very organs where leukaemia virus replicates; nevertheless, in order to obtain antiviral effects it is necessary to apply the analogue daily. This suggests that the polymer is gradually segregated in organs into a group of cells or into subcellular organelles away from the primary sites of viral replication. In turn these results suggest that for directly acting polymeric drugs a half life over 24 hours is without advantage[40].

In conclusion, it is necessary to emphasize that the compounds described here represent only a segment of all known polymeric analogues of nucleic acids. Compounds of this type were first prepared at the University of Birmingham in in the 1950s by A. S. Jones and his collaborators and have been studied at a moderate rate ever since; their chemistry has been recently reviewed[48]. Polymers similar to those described here, but carrying any desired electric charge and having any desired stability can be synthesized if there are compelling reasons for doing so. These reasons can be created only by a systematic study of the biological properties of polymers; it is hoped that the present work is a valid step in that direction.

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Note added in proof

Recently the preparation of poly(5-vinyluracil) has been described (Jones, A. S. and Walker, R. T. *Nucleic Acid Research Special Publication* 1976, **1**, 1). Polymer is sparingly soluble in water and does not hybridize with poly(adenylic acid).