Effect of Synthetic and Biological Polyanions on Herpes Simplex Virus.* (29098)

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In view of our recent findings that heparin had an inhibitory effect on herpes simplex virus(1), various synthetic and other biological polyanions were investigated for similar activity. To ascertain possible relationships of such compounds to this inhibitory effect, the following characteristics were studied: nature, size and branching of the polyanionic molecule; nature and amount of negative groups; and relative importance of the polysaccharide portion. The biological preparations, hyaluronic acid, keratosulfate, chondroitin sulfates A, B, and C were also tested because of their possible role as a host defense mechanism in viral infection.

Materials and methods. Virus. A laboratory strain of herpes simplex virus (Rodanus) which had undergone multiple passages in eggs and primary human amnion tissue cultures, was employed for all experiments. (Seven other strains of this virus, including 4 recent clinical isolates, had previously been found to be similarly inhibited by heparin.)

Tissue culture and media. Primary human amnion cultures were prepared and maintained with bovine amniotic fluid media(2).

Substances tested:

A. *Biological polyanions*

1. *Heparin.* Panheparin solution (Abbott #2945—Chicago, Ill.), 20,000 USP units/ml, 100 units/mg, containing no preservatives. (Five other heparin preparations from 3 different sites of pork and beef material, obtained from 3 different companies, had previously been found to exhibit a similar inhibitory effect on herpes simplex virus.)

2. *Keratosulfate* (M VI 52 IV)†

3. *Chondroitin sulfate* A (M VII 34 III)†

4. *Chondroitin sulfate* B (M VII 33 AI)†

5. *Chondroitin sulfate* C (M V 98 II)†

6. *Hyaluronic acid*—sodium salt from human umbilical cord. (a) from Sigma Chemical Co., (b) from Dr. E. Balazs, Retina Foundation, Boston.

B. *Nonbiological polyanions* (Table I). With the exception of sulfopolyglucin, all polysaccharide sulfates were prepared from the corresponding polysaccharides by sulfation with chlorosulfonic acid in pyridine(3).

1. *Sulfopolyglucin.* (Riker Lab., Northridge, Calif.)—sodium salt of a sulfated low molecular weight polyglucose molecule.

2. *Potato amylopectin* ("Ramalin"), contributed by Dr. Max Goldfrank, from the Stein, Hall and Co., New York.

3. *Corn amylopectin* with a degree of polymerization above 2,000, prepared by the method of Schoch(4). Corn amylopectins of low degree of polymerization ("SHAP" Series) were obtained by mild acid hydrolysis (3).

4. *Corn amyloses.* A product (see SAM VII) with a degree of polymerization of about 800, obtained by Schoch's method(4). Polysaccharides with lower molecular weights, such as in SAM III and IV, were products of careful fractional extraction of corn starch prepared and described earlier(5).

5. *Dextrans* were obtained through the courtesy of Dr. Allene Jeanes,‡ who also provided the results of her analyses of these products.

6. *Polyvinyl sulfate* (K-salt), obtained by sulfation of a commercial sample of polyvinyl alcohol, Elvanol 71/24 from duPont de Nemours and Co.

7. *Polystyrene sulfonate,* Lustrex X770

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‡ Northern Utilization Research and Development Division, U. S. Dept. of Agri., Peoria, Ill.

TABLE I. Effects of Various Polymers on Herpes Simplex Virus.

Polymer	No. of sulfate groups/repeating unit	Degree of polymerization	Degree of branching	Min inhib conc (μg/ml)	Heparin index
A1. Heparin	2.5†	45-55	—	10	1
B1. Sulfopolyglucin	2.5-3*	12-14	—	100	.1
B2. Potato amylopectin	—	>6000	2-4% (α-1,6)	No inhib at 1000 μg/ml	No inhib
Sulfated potato amylopectins (a) SAP 23	.17*	>6000	*Idem*	250	.04
(b) SAP 25	1.01*	>6000	"	7.5-10	1-1.5
B3. Corn amylopectin	—	>2000	"	No inhib at 1000 μg/ml	No inhib
Sulfated corn amylopectins					
(a) SAP 21	.57*	>2000	"	5 -7.5	1.5- 2
(b) " 16	.85*	>2000	"	5	2
(c) " 36	1.25*	>2000	"	2.5-5	2 - 4
(d) " 33	1.41*	>2000	"	2.5-5	2 - 4
(e) " 47	2.06*	>2000	"	2.5-5	2 - 4
(f) " 45	2.18*	>2000	"	2.5-5	2 - 4
(g) SHAP II	1.72*	405	"	2.5-5	2 - 4
(h) " III	2.22*	105	"	2.5-5	2 - 4
B4. Sulfated corn amylose					
(a) SAM VII	2.22*	800	0	2.5-5	2 - 4
(b) " IV	1.44*	161	0	2.5-5	2 - 4
(c) " III	1.64*	80	0	2.5-5	2 - 4
B5. Sulfated dextrans					
(a) S-DEX III	1.44*	240,000	5% (α-1,3)	2.5-5	2 - 4
(b) " IV	1.58*	540	5% (α-1,3)	2.5-5	2 - 4
(c) " V	1.59*	>6000	7% (α-1,4)	2.5	4
B6. Polyvinyl sulfate (SEL 3)	.58	—	—	1-2.5	4 -10
B7. Polystyrene sulfonate (Lustrex X770)	.64	—	—	2.5	4

* Hexose unit. † Disaccharide unit.

(Na-salt), kindly supplied by Monsanto Chemical Co., Springfield, Mass. This product is no longer commercially available.

Estimation of inhibitory effect of test substance. A 30-300 TCD_{50} dose of virus in 0.3 ml volume, prepared with Hanks' BSS as diluent, was added to 0.3 ml of an appropriate dilution of the test substance in saline or phosphate buffer (pH 7.2). The viral-test substance mixture was incubated for 1 hour at 4°C and 0.2 ml was inoculated into each of 2 tissue culture tubes. Control tubes without test substance and containing an equivalent amount of virus were included in the experiment. Cytopathogenic effects (CPE) were recorded every 2 or 3 days. No medium change was made and tubes were discarded after 2 weeks. Each test substance was evaluated at least twice by this technique. The minimum inhibitory concentration (MIC) of a test substance is reported in micrograms per ml of final tissue culture medium. The inhibitory potency of the various preparations tested is compared with that of heparin in Table I (Heparin Index). In view of the slight variation in MIC in duplicate experiments, the MIC and heparin index are given as a range in some instances.

Results. Characteristics of polymers as related to viral inhibitory potency (Table I).

(a) *Degree of sulfation.* Unsulfated polysaccharides (compounds B2 and B3) produced no inhibition. With amylopectins of low sulfation (compds. B2a and B3a), inhibitory effect was noted only at higher concentrations. The heparin index reached a plateau at a ratio of about one sulfate group per hexose; above this point no further increase in viral inhibition was observed.

(b) *Size of molecule.* Although sulfopoly-

glucin (compd. B1) is a highly sulfated molecule, its heparin index was found to be significantly lower than that of heparin or of other sulfated polymers. This difference may be attributed to its smaller molecular size (12-14 glucose residues per molecule). On the other hand, amylose sulfate with 80 glucose residues per molecule (compd. B4c) has the same heparin index as a homologous product with 10 times as many glucose residues per molecule (compd. B4a). No difference in inhibitory potency can be detected among homologous products over a wide range of molecular weights, either in the amylopectin sulfate (compds. B2 and B3) or the dextran sulfate (compd. B5) series.

(c) *Degree of branching.* There was no apparent difference between the branched sulfated corn amylopectin compounds and the linear sulfated corn amylose polymers, even at similar molecular weights and degrees of sulfation (*e.g.,* compds. B3c-h and compds. B4a-c).

(d) *Influence of glycosidic bonds.* There was no significant difference in inhibitory potency between sulfated amylopectins (B3 series with α-1,4 bonds and α-1,6 branching bonds) and dextrans (B5 series with α-1,6 bonds and α-1,3 or α-1,4 branching bonds). This demonstrates that the type of glycosidic bond has no influence on viral inhibition.

(e) *Chemical nature of polymer.* The polysaccharide portion of the sulfated polysaccharides tested does not appear to contribute to the inhibitory effect since similar viral inhibitory activity was found to be exerted by polyvinyl sulfate (compd. B6) and polystyrene sulfonate (compd. B7). These chemically unrelated synthetic polymers have in common with the polysaccharide sulfates only their macromolecular nature and the presence of sulfate ester or sulfonic groups.

(f) *C-O-S (sulfate)* versus *C-S (sulfonic) bond.* No significant differences were noted between polystyrene sulfonate, which contains a carbon to sulfur bond, and polyvinyl sulfate and sulfated polysaccharides which contain ester bonds. The presence of the C-N-S bond, in addition to the C-O-S bond, as found in heparin, did not appear to exert any additional effect.

Failure of biological acid mucopolysaccharides to affect herpes simplex virus. In addition to heparin, the following biological acid mucopolysaccharides were tested: keratosulfate, chondroitin sulfates A, B, and C, and hyaluronic acid from two sources. None of these preparations were found to exhibit a viral inhibitory effect in final concentrations of 100 or 1000 μg/ml. These concentrations are 10-100 times greater than the concentration of heparin needed for inhibition (Table I).

Discussion. The effects of various polyanions on viruses have been reported by several workers(6-9). Detailed studies in other systems(6,9) suggest that these substances influence the primary electrostatic attachment of virus to cell prior to viral penetration.

Our results with the polymers tested confirm the concept that the viral inhibition is due primarily to the strong electronegative charges of the polyanion. Within limits, this effect is dependent upon the degree of sulfation of the molecule (*i.e.,* on the amount of negative charges). When one sulfate group per hexose unit is present in the sulfated polysaccharides, a maximum effect is reached. Carboxyl groups (*e.g.,* hyaluronic acid) cannot replace the strongly negative sulfate groups.

The inhibitory effect appears to require a minimum size of the molecule (degree of polymerization) as demonstrated with sulfopolyglucin. The inhibitory effect is independent of (1) the existence or absence of branching of the molecule, (2) the type of glycosidic bonds, and (3) the type of sulfur bond (sulfonic or sulfate ester). The polysaccharide portion of the molecule does not appear to be essential, since synthetic polymers, such as polyvinyl alcohol or polystyrene sulfonate, could be substituted for it.

At the concentrations tested, no inhibitory effect was noted with biological acid mucopolysaccharides, other than heparin, *i.e.,* hyaluronic acid, keratosulfate and the chondroitin sulfates A, B, and C. It should be noted that the first mentioned contains no sulfate groups at all, whereas the others have lower sulfate content than heparin (about 0.5 sul-

fate groups per hexose in the chondroitin sulfates, or one per repeating unit, as opposed to 1.25 sulfate per hexose in heparin or 2.5 per repeating unit). It is of interest that amylopectin sulfates with as little as 0.57 and 0.17 sulfate per hexose, have distinct inhibitory potency, whereas the chondroitin sulfates with about 0.5 sulfate per hexose are without effect. This difference might be due to interference by either N-acetylhexosamine or carboxyl groups with the inhibitory potency of the polymer. An analogous situation has been encountered in the interaction of various polyanions with human serum beta-lipoproteins(3).

The infrequency with which viral infections of joints, cartilage, bone and other connective tissues have been reported, may be related to a protective effect afforded by the high content of acid mucopolysaccharides present in these tissues. This effect may be due not only to the negative charge which these compounds possess, but also to their capacity to serve as filtering barriers to virus particles. The latter concept is supported by the observations, reviewed by Duran Reynals (10), that subcutaneous injections of hyaluronidase can induce extension of local dermal viral infections to deeper tissue layers. In this regard, the *in vitro* finding(11) that larger sized particles (30 mμ) are delayed in their passage through hyaluronic acid may also be pertinent.

Summary. Several synthetic and biological sulfated polymers were investigated for their inhibitory effect on herpes simplex virus in tissue culture. Of the biological preparations tested, heparin was the only one which inhibited this virus. Nineteen other sulfated polymers were assayed and found to inhibit herpes simplex to varying degrees. The inhibitory potency of the compound depended within limits on the degree of sulfation and on the size of the molecule. The viral inhibitory effect was independent of the polysaccharide portion or branching of the molecule, and of the type of glycosidic or sulfur bond.

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Characterization of Serum Amylase Activity in Normal and Pancreatectomized Dogs. (29099)

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Isoenzymes of amylase have been reported in sera of various experimental animals as well as man(1-7). These variant forms of amylase presumably are elaborated more or less specifically by certain tissues, but their exact sites of origin are poorly delineated. In the hope of extending available information on these points, certain properties and enzyme kinetics of electrophoretic and chromatographic fractions of serum amylase were investigated in normal and pancreatectomized dogs.

Materials and methods. Blood was obtained from 9 normal dogs and from 6 dogs