

Antiviral Activity of Polyacrylic and Polymethacrylic Acids

I. Mode of Action in Vitro.

P. DE SOMER, E. DE CLERCQ,¹ A. BILLIAU,¹ E. SCHONNE, AND M. CLAESEN

Rega Institute for Medical Research, University of Leuven, Leuven, Belgium

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Polyacrylic acid (PAA) and polymethacrylic acid (PMAA) were investigated for their antiviral properties in tissue culture. Compared to other related polyanions, as dextran sulfate, polystyrene sulfonate, polyvinyl sulfate, and polyphloroglucinol phosphate, PAA and PMAA were found to be significantly more antivirally active and less cytotoxic. PMAA added 24 hr prior to virus inoculation inhibited viral growth most efficiently but it was still effective when added 3 hr after infection. Neither a direct irreversible action on the virus nor inhibition of virus penetration into the cell could explain the antiviral activity of PMAA. PMAA inhibited the adsorption of the virus to the host cell and suppressed the one-cycle viral synthesis in tissue cultures inoculated with infectious RNA.

Cationic and anionic polyelectrolytes of both natural and synthetic origin exhibit a potent inhibitory effect on viruses, bacteria, tumors and enzymes.

Schematically, the virus particle may be attacked in one or more of its four situations: (i) outside the cell, (ii) attached to the cell surface, (iii) penetrating into the host cell, (iv) in the interior of the cell or leaving the cell. The antiviral effect of polycationic and polyanionic substances occurs during the early interaction of virus and cells: polycations such as lysine polypeptide inhibit the infectivity of influenza APR8 (22), infectious bronchitis virus, Newcastle disease virus (10), mumps virus (9), and tobacco mosaic virus (3) by direct interaction with the virus particle. Polyanions, however, combine both with viruses [e.g., acid mucopolysaccharide (16), polyphloroglucinol phosphate (32), agar polysaccharide (24), and heparin (25, 30)] and receptor sites on the cell surface [e.g., polyglutamate, polyacrylate and polypectate (26), ethylene anhydride copolymers (7), and agar polysaccharide (27)].

Some polyanions, such as dextran sulfate and ethylene maleic anhydride copolymers, also act by blocking intracellular virus replication (7, 27). The present report describes the antiviral activity and the mode of action of the acrylic and methacrylic polyanions in vitro.

¹ "Aspirant" of the Belgian N.F.W.O.

MATERIALS AND METHODS

Tissue culture. A second passage of primary rat embryo fibroblasts (REF) and primary rabbit kidney (PRK) cells was employed in our experiments. Continuous cell lines of baby hamster kidney (BHK21) and HeLa cells were also used.

Culture media. Eagle's medium enriched with 0.5% lactalbumin hydrolysate and 10% inactivated calf serum was used. Upon virus inoculation, only 3% calf serum was included.

Viruses. Vesicular stomatitis virus (VSV, Indiana strain) was maintained and titrated on mouse embryo fibroblasts. Sindbis virus (strain Egypt AR 339) was propagated in chick embryo fibroblasts and titrated on REF. Influenza APR8 underwent serial passages in the chorioallantoic cavity of chick embryos and was titrated in embryonated eggs; the appearance of hemagglutinin was used as the criterion of infection (EID_{50}). Vaccinia virus and herpes simplex virus were maintained on HeLa cells and underwent one passage in REF prior to use. Vaccinia was titrated on REF and herpes simplex was titrated on PRK. Poliovirus type 1 was propagated and titrated on HeLa cells.

Infectious Sindbis and polio ribonucleic acid (RNA) were prepared from concentrated virus stocks according to the cold phenol extraction method described by Wecker and Schäfer (33). Both stocks contained $5 \cdot 10^8$ plaque-forming units (PFU) per ml. The RNA preparations were diluted 1:2 in 2.0 M MgSO_4 and 10^{-2} M tris(hydroxymethyl)aminoethane (Tris) buffer, pH 7.2, and applied on monolayers of HeLa cells or REF as mentioned below.

Polymethacrylic acid (PMAA). A 15% solution of distilled methacrylic acid in benzene was polymerized

at 67 C for 4 hr in the presence of 0.1% benzoylperoxide. The polymer was precipitated, purified twice by dissolution in methanol and precipitation with ether, and dried in vacuo at 40 C.

For the fractionation (34), 12.5 g of polymethacrylic acid was dissolved in 750 ml of dry methanol; methylisobutyl ketone was added at 26 ± 0.1 C until a precipitate appeared. The precipitate was redissolved by warming to 35 C, and the solution was cooled slowly to 26 C in a thermostat. The precipitate was collected after 1 day and redissolved in methanol. Reprecipitation was accomplished by pouring the solution into 10 volumes of ether. The polymer was isolated and dried in vacuo at 65 C. In this way, 11 fractions were obtained. The molecular weight was calculated from viscosity data determined in anhydromethanol at 26 C (34). The first fraction with the highest molecular weight was discarded. The other fractions had a molecular weight between 25,000 and 1,175,000.

Polyacrylic acid (PAA). A 56-g amount of freshly distilled acrylic acid, dissolved in 200 ml of purified dioxane, was polymerized in nitrogen atmosphere at 70 C for 4 hr in the presence of 200 mg of benzoylperoxide. The viscous suspension was diluted with 300 ml of dioxane and poured into 3 liters of dry benzene. The gummy substance was dissolved in methanol and precipitated by pouring the solution into ether. This operation was repeated, and the polymer was finally obtained in a solid state (45 g) by lyophilization of a solution in dioxane.

The polyacrylic acid was fractionated by a technique similar to that described for the polymethacrylic acid. By progressive addition of *n*-heptane to a solution of 15 g of the polymer dissolved in 1.5 liters of dioxane, 10 fractions were obtained. The first two fractions, which contained most of the cross-linked polymer, were discarded. The viscosity was determined at 30 C in dioxane (19). The molecular weight ranged from 27,000 to 1,050,000.

Polystyrene sulfate was prepared by interaction of 18 g of polystyrene in 200 ml of pyridine with 12.5 ml of sulfonyl chloride and precipitation of the reaction product with four volumes of saturated NaCl.

The other polymers, dextran sulfate 500, sodium salt (Pharmacia, Uppsala, Sweden), polyvinyl sulfate, potassium salt, (Serva, Heidelberg, Germany), polyphloroglucinol phosphate, sodium salt (No. 137a, Leo Co., Hälsinborg, Sweden), were commercially available.

RESULTS

Preliminary studies. Preliminary studies had shown that antiviral activity of PMAA and PAA increased proportionally with increasing molecular weight: PMAA and PAA achieved maximal reduction of Sindbis virus cytopathogenicity in REF monolayers at the higher molecular weights (approximately 600,000 and 1,000,000, respectively). For this reason, PMAA and PAA fractions of the indicated molecular weights were used throughout our experiments.

Compared activity of PMAA and related polyanions in various virus-cell systems. The VSV-PRK, herpes-PRK, Sindbis-REF, and vaccinia-REF virus cell systems were chosen to compare the antiviral activity of PMAA, PAA, and other representative polyanions as sodium dextran sulfate, polystyrene sulfonate, sodium polyvinyl sulfate, potassium polyvinyl sulfate, and polyphloroglucinol phosphate. The tissue culture was exposed to various concentrations of polymer (100, 10, 1, and 0.1 µg/ml) and inoculated 24 hr later with 100 TCID₅₀ of the appropriate virus challenge. Antiviral activity and cytotoxic effect were determined (Table 1).

PMAA and PAA were especially effective against vaccinia. PMAA achieved greater inhibition of VSV and Sindbis cytopathogenicity than did PAA. Herpes simplex was not sensitive either to PMAA or to PAA. The antiviral action of dextran sulfate was limited to VSV and herpes simplex. Polystyrene sulfonate was entirely inactive. Dextran sulfate and polyvinyl sulfate prevented cytopathogenicity at concentrations closely related to concentrations injuring normal cell morphology.

Unlike dextran sulfate, polyvinyl sulfate, and polyphloroglucinol phosphate, which prevented cell attachment to glass and caused retraction to clumps (28, 30), PMAA and PAA did not alter

TABLE 1. Protective effect of PMAA and related polyanions against viral cytopathogenicity in cell cultures

Polyanions	Virus ^a cell system			
	VSV/PRK	Herpes/PRK	Sindbis/REF	Vaccinia/REF
PMAA	1-10 ^b	>100	0.1-1	0.1-1
PAA	10-100	>100	10	0.1-1
Dextran sulfate	1-10	1-10	>100	>100
	10 ^c	10 ^c	100 ^c	100 ^c
Polystyrene sulfonate	>100	>100	>100	>100
Polyvinyl sulfate	10	1-10	10-100	10-100
	10 ^c	10 ^c	10 ^c	10 ^c
Polyphloroglucinol phosphate	10	ND ^d	ND	1
	10 ^c			100 ^c

^a Virus input: 100 TCID₅₀.

^b Minimal inhibitory concentration (µg/ml): concentration of polymer required to afford a minimal inhibition of CPE (reduction of viral cytopathogenicity by 25%).

^c Threshold concentration of polymer which revealed toxic alteration of cell morphology.

^d Not done.

the general cell morphology at concentrations up to 1 mg/ml.

Effect of time of addition of PMAA. Fully grown REF monolayers in tissue culture tubes were inoculated with 30 TCID₅₀ of Sindbis virus per tube. After 30 min of adsorption at 4 C, the unadsorbed virus was removed and replaced by maintenance medium. PMAA was added to the medium at a final concentration of 100 µg/ml at different times prior to and after infection. Once exposed, the culture remained continuously in contact with the polymer. After 0, 12, 24, 48, and 72 hr after infection, a series of four culture tubes was harvested and tested for total virus content.

PMAA caused a marked reduction in virus yield, proportional to the length of time of exposure (Fig. 1). When applied as late as 3 hr or immediately after viral inoculation, PMAA assured serious inhibition of Sindbis virus multiplication; more reduction of virus yield occurred when PMAA was added 24 hr or just before infection.

Since inhibition of virus production was most pronounced with PMAA treatment starting 24 hr

prior to infection, PMAA was added 24 hr before virus inoculation in the remainder of our tests.

Virus inactivation. To test the possibility of a direct interaction with the virus particles, 10⁷ PFU of VSV or 10⁸ EID₅₀ of influenza APR8 were mixed with 5 mg of PMAA per ml in maintenance medium and incubated at 4 C for 16 hr. Control virus preparations free from compound were included. The initial and residual virus contents were determined by titrating the VSV samples in PRK culture tubes and the influenza APR8 samples in the chorioallantoic cavity of chick embryos. Interaction of residual PMAA in the virus assay procedure must not be considered, since the polymer was diluted "out" beyond its active concentration level. The results in Table 2 show the difference in residual infectivity of VSV and influenza APR8, incubated in the presence and absence of PMAA. No measurable virus inactivation was obtained with a concentration of compound which provided a marked antiviral activity in tissue culture.

Effect on virus adsorption. The effect of PMAA on virus adsorption was measured by the disap-

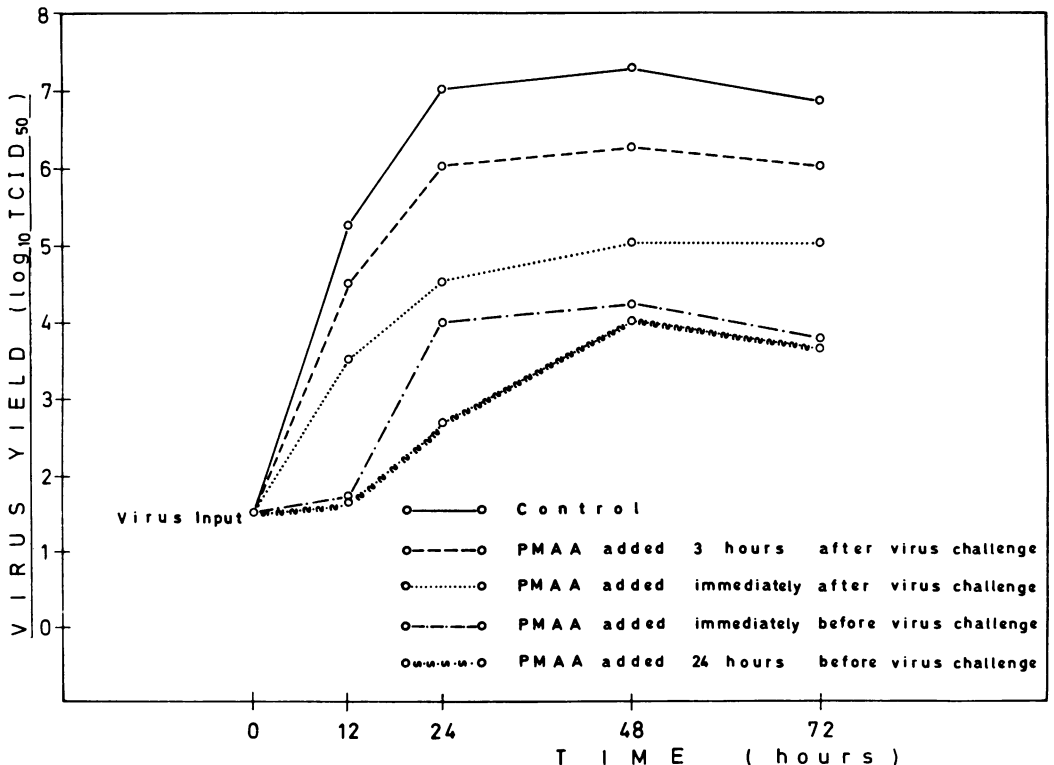


FIG. 1. Inhibition of Sindbis growth in REF treated with PMAA. Confluent monolayers were inoculated with 30TCID₅₀ Sindbis virus per tissue culture tube. At different times prior to and after infection 100 µg/ml of PMAA was added.

TABLE 2. *Virucidal activity of PMAA-virus infectivity after incubation in the presence or absence of PMAA*

Virus	With PMAA	Without PMAA
VSV.....	4.8 ^a	4.6 ^a
APR8.....	7.9 ^b	7.8 ^b

^a Infectivity expressed in log₁₀ TCID₅₀/ml.

^b Infectivity expressed in log₁₀ EID₅₀/ml.

pearance of virus from the medium bathing the cell cultures. PRK monolayers in 60-mm plastic petri dishes were exposed to 100 µg of PMAA per ml for 24 hr. Treated and untreated cultures were washed throughout with medium lacking the polyanion and inoculated with different VSV doses (Table 3). Virus adsorption was carried out at 4 C. Attachment of virus to cells was almost independent of temperature between the range of 0 to 4 C and 36 C (2, 14). The rate of adsorption was influenced only by temperature, as far as could be expected from the degree of viscosity of the medium (1). At 4 C (12, 29), the penetration process was strongly depressed and might be disregarded at this low temperature.

After 1 hr of incubation at 4 C, the culture media containing the unadsorbed virus were collected. The remaining infected cell sheets were covered with a starch overlay (4) and further incubated at 36 C; after 1 to 2 days, the virus plaques were treated with neutral red stain and counted. The culture media containing the unadsorbed virus were also assayed for plaque-forming virus by means of the starch overlay technique. A dilution (1 ml at 1:20) was applied to the PRK cells grown in petri dishes and incubated for 1 hr at 36 C, with gentle moving and 5% CO₂ supply. The supernatant fluid was then replaced by starch overlay and further titration as described previously (Table 3).

The amount of adsorbed virus was significantly reduced when cell cultures were pretreated with PMAA. The inhibition reached 80% for 5,000 PFU virus input to 90% for 200 PFU virus input. Secondly, about 80 to 90% of the inoculated virus was recovered in the medium of cell cultures exposed to PMAA, whereas only 10 to 20% of the virus remained in the supernatant fluid of the control cultures. The reduction in the number of virus plaques in cells pretreated with PMAA was largely compensated for by a proportional increase of unadsorbed virus particles in the supernatant fluid. These results indicated that, under the concrete experimental conditions, the reduction of virus infectivity must be due to a suppression of virus adsorption.

The effect of PMAA on the adsorption of

Sindbis virus to REF was determined under similar conditions. An identical though less pronounced effect was obtained. The Sindbis plaque formation was reduced by only 50%; this decreased plaque number could be fully attributed to inhibition of the adsorption process, since the disappearance of virus from the supernatant fluid was also reduced by 50%.

Effect on virus penetration. Penetration refers to the sequence of events starting with irreversible attachment of the virus particle on the cell membrane (20). As long as it is bound to the cell membrane, the virus may be inactivated by antiviral antibody (12, 29). Once it has penetrated into the cell, it escapes from the antibody influence. If penetration is blocked, either by low temperature (4 C) or by antiviral substance, the virus attached to the cell surface remains susceptible to inactivation by antibody for a much longer period than do viruses at 37 C in a compound-free medium.

Thus, if PMAA was acting on the penetration process, the addition of specific antiserum must provide a significantly greater inhibition of virus production in the PMAA-treated cultures than in the untreated ones. Full-grown REF monolayers in culture tubes were infected with Sindbis virus at an input multiplicity of 10⁻¹ TCID₅₀ per cell. After 15 min of incubation at 4 C, the unadsorbed virus was removed by washing the tissue culture

TABLE 3. *Effect of PMAA on adsorption of VSV to PRK cells*

Total virus amount ^a (adsorbed and unadsorbed in control samples; PFU/ml)	Amount of adsorbed virus ^b (PFU/ml)		Amount of unadsorbed virus ^c (PFU/ml)		Total virus amount ^a (adsorbed and unadsorbed in PMAA samples; PFU/ml)
	With PMAA	Without PMAA	With PMAA	Without PMAA	
5,000	676 ^d	4,000	4,980	1,000	5,656
5,000	700	4,000	4,320	1,000	5,020
1,388	864	1,280	828	108	1,692
1,442	772	1,256	780	186	1,552
790	114	730	384	60	496
849	122	783	546	66	668
208	109	202	126	6	235
172	30	148	156	24	186

^a Total amount of virus resulting from the addition of both adsorbed and unadsorbed virus fractions is expected to be identical in both pretreated and untreated cultures.

^b Amount of adsorbed virus is determined in plaque-forming units for 1 ml of the original virus suspension.

^c Determination of the amount of unadsorbed virus is based on the plaque count obtained for 1 ml of 1:20 dilution.

^d Each value corresponds to the mean obtained for two petri dishes.

with Eagle's medium. Compound-treated monolayers received 100 μg of PMAA per ml from 5 min to 2 hr after infection. Antibody-treated monolayers received a 1:40 dilution of a specific Sindbis rabbit antiserum from 1.5 to 2 hr post-infection. Infected cell cultures were harvested several times (Fig. 2) and assayed for total virus content in BHK21 cells. When administered separately, both PMAA and antiserum caused a significant reduction of virus growth. The combination of antiserum plus PMAA resulted in a simple addition of both effects; antiserum did not provide a more marked virus yield reduction in PMAA-treated tissue cultures than in untreated ones. There was no evidence that, in the presence of PMAA, adsorbed virus was retained on the cell surface and was inhibited from penetrating into the cell.

Effect on virus maturation and release. When added after the adsorption of virus to the cells, PMAA was still able to reduce the virus production. Roughly, this inhibition could result from an effect on penetration, maturation, or release of virus. Since PMAA did not impair virus penetration, it must affect the multiplication of intracellular virus or release of virus from cells. This hypothesis was tested in the following experiment.

It is generally accepted that viruses cannot grow in cells which lack the necessary receptors for the viral protein. However, when released from its protein coat, the viral RNA can enter the cells and form complete virus particles. As resulting particles are unable to infect other cells, only a single-cycle growth occurs. Such a system was

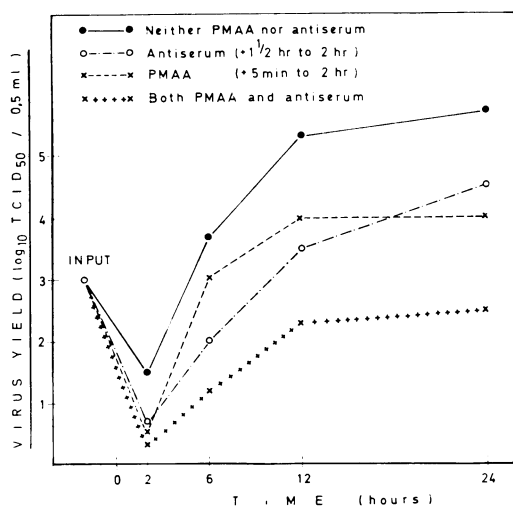


FIG. 2. Effect of PMAA, antibody, and combined PMAA-antibody treatment on the production of Sindbis in REF.

suitable for the study of the specific effect of PMAA on the adsorption, penetration, transcription, and translation of the virus nucleic acid and on the release of complete virus. In a preliminary experiment, HeLa cells were found to be unsusceptible to Sindbis virus, just as REF were to poliovirus. The Sindbis and poliovirus RNA preparations were diluted 1:2 in 2.0 M MgSO₄ containing 10⁻²M tris(hydroxymethyl)aminoethane (Tris) buffer at pH 7.2. PRK cells in tissue culture tubes were washed with 1.0 M MgSO₄ in 10⁻² Tris buffer and incubated with 0.2 ml of the RNA preparation for 20 min. The inoculum was removed and the cells were washed with 2 ml of phosphate-buffered saline. One ml of maintenance medium was added, and the tubes were incubated at 36 C until harvest. Compound-treated monolayers were exposed to 100 μg or 10 μg of PMAA per ml from 24 hr before to 3 hr after infection. The virus inoculum, Tris buffer, and saline wash also contained 100 μg or 10 μg of the compound per ml. Sindbis and poliovirus were harvested by freeze-thawing 24 hr after RNA challenge and titrated in REF and HeLa cells, respectively.

A marked inhibition of Sindbis production was observed in monolayers treated with PMAA (Table 4). On the other hand, the multiplication of poliovirus was rather enhanced. It seemed, therefore, that inhibition of Sindbis RNA by PMAA must be considered as a specific phenomenon, not valid for all kinds of infectious RNA. Further evidence for the interaction of PMAA with the Sindbis virus RNA was provided by a stepwise analysis of the Sindbis virus growth during the 24-hr postinfection period. Compound-treated cell cultures received 100 μg of PMAA per ml from 24 hr before to 3 hr after infection. PMAA-treated and untreated monolayers were

TABLE 4. Effect of PMAA on the one-cycle multiplication of Sindbis-RNA and poliovirus-RNA in HeLa cells and REF, respectively^a

Polyanions	Sindbis-RNA (in HeLa cells)	Poliovirus-RNA (in REF)
Control	4,800 ^b	10 ^{4.13c}
PMAA (10 $\mu\text{g}/\text{ml}$)	290 ^b	10 ^{4.45c}
PMAA (100 $\mu\text{g}/\text{ml}$)	440 ^b	10 ^{5.0c}

^a Compound-treated cell cultures were exposed to PMAA from 24 hr before to 3 hr after infection.

^b Sindbis virus yield in HeLa cells determined 24 hr after viral RNA challenge by titration in REF in petri dishes and expressed in plaque-forming units per ml.

^c Poliovirus yield in REF determined 24 hr after viral RNA challenge by titration in HeLa cells in tissue culture tubes and expressed in TCID₅₀/ml.

harvested 8, 12, 16, or 24 hr after Sindbis-RNA inoculation and titrated on BHK21 cells. The results (Fig. 3) showed a striking yield reduction throughout the whole first cycle of virus multiplication.

DISCUSSION

Whereas dextran sulfate, polyvinyl sulfate, and polyphloroglucinol phosphate exerted a potent inhibitory effect on herpes simplex infectivity in cell cultures [Takemoto and Fabish (27), Vaheri (30), Nahmias et al. (18), and Takemoto and Spicer (28)], PMAA and PAA were more specifically active on VSV, vaccinia, and Sindbis virus.

Moreover, the viral inhibitory efficiency of sulfate polyanions (polyvinyl sulfate, dextran sulfate) appeared to correlate with an inhibition of cell attachment to glass and formation of cell aggregates or cell clumps (18, 23, 27). Since PMAA did not prevent cell attachment and did not interfere with normal cell growth, its antiviral activity was due not to a general nonspecific effect on cells but to a direct interaction with virus replication.

The inhibition of viral growth was dependent upon the time of addition of PMAA. The earlier the tissue cultures were exposed to the polymer, the greater the reduction in virus yield. Although PMAA has been found most effective when added 24 hr prior to virus inoculation, it remained partly active when added after infection.

PMAA did not inactivate the virus particle in its extracellular state, thus supporting the view that PMAA did not combine irreversibly with the virus particle as did certain polyanions such as polyphloroglucinol phosphate, polyvinylalcohol phthalate, copolymers of styrol and maleic acid,

and formaldehyde polymers of phenolsulfonic acid (31). It was, however, quite possible that PMAA exerted a reversible effect on the virus particle. Acid mucopolysaccharide, heparin, and dextran sulfate would inhibit Theiler's encephalomyelitis virus, encephalomyocarditis, and herpes simplex in this way (15-17).

Direct interference with the virus particle, or a combined action on the virus and the cell receptor sites, could account for the inhibitory effect of PMAA on the virus attachment to the host cell. Both cell surface and virus particle are occupied by predominantly negative charges (8). Adsorption is thought to be achieved by interaction between the amino groups of the virus and the phosphate groups of the cell wall (1). Repellent forces between both negatively charged interacting surfaces prevent the virus from coming sufficiently close to the cells; raising the cation concentration in the suspending medium aids in overcoming this electrostatic barrier. Up to a certain concentration, cations and polycations enhance the adsorption of fowl plaque virus by chick embryo cells (1). Polyanions, on the other hand, suppress adsorption, presumably owing to a reinforcement of the net negative charge of both cells and viruses. Similarly, PMAA would block virus adsorption by enhancing the repellent forces between the interacting cells and viruses. It is not unlikely that PMAA would also affect the virus-cell attachment by spreading a three-dimensional gel network over all of the cell surface. Hyaluronic acid and ethylene maleic anhydride copolymer would impair the virus cell binding by a similar macromolecular sieve system (7, 13).

The study of the effect of PMAA on the growth of infectious viral RNA in unresponsive cells allowed us to exclude the role of the cell receptors for viral proteins. Inhibition of virus production by PMAA in this system must be interpreted as resulting from: (i) direct inactivation of viral RNA, (ii) interaction with the uptake of RNA by the cell, (iii) inhibition of RNA replication into the cell, or (iv) release of complete virus from the cell. At first, one could imagine that polyanions such as PMAA hamper the likewise negatively charged nucleic acids in reaching the cell surface, since polycations such as diethylaminoethyl-dextrane stimulate the entry of infectious nucleic acid into the host cell (6, 20, 31). Secondly, PMAA could affect some metabolic reactions required for viral synthesis and release. The finding that PMAA exerts a marked inhibition on Sindbis-RNA, leaving poliovirus RNA intact, argues for a specific interaction with the RNA metabolism, possibly on the intracellular stage of virus replication.

The possibility that the antiviral action of

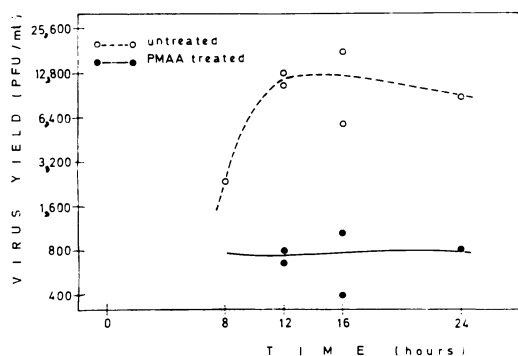


FIG. 3. Effect of PMAA on the one-cycle multiplication of Sindbis-RNA in HeLa cells. Compound-treated monolayers were exposed to 100 μ g of PMAA per ml from 24 hr before to 3 hr after infection. Sindbis virus yield was determined 8, 12, 16, and 24 hr after viral RNA challenge by titration on BHK21 cells in petri dishes and expressed in PFU per ml.

PMAA on the intracellular level is mediated by induction of interferon has been raised, since the PMAA has been found to stimulate the production of interferon *in vivo* (5). The applied method is assumed to be the most sensitive of the available assay methods for detection of interferon (11). Yet we failed to demonstrate the presence of interferon in the frozen and thawed cell homogenates. Nevertheless, it remains conceivable that interferon is being produced in minimal amounts sufficient to block the virus multiplication within the same cell where it is induced, but insufficient for dissociation from the cellular structures and for detection in the supernatant fluid by the usual laboratory methods.

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