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Antiviral Activity of Polyacrylic and Polymethacrylic Acids

II. Mode of Action in Vivo.

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A marked virus-inhibiting potency is obtained in the serum after intraperitoneal injection of polyacrylic acid (PAA) and polymethacrylic acid (PMAA) in mice. Much higher antiviral levels were reached than for other related polymers including dextran sulfate, heparin, polyvinyl sulfate, pyran copolymer, polystyrene sulfonate, and macrodex. The broad antiviral action of PAA and PMAA was attributed both to a direct interference with the virus-cell interaction and the viral ribonculeic acid metabolism and to the formation of an interferon-like factor. Both polyanions differed in interferon-inducing ability: highest serum interferon titer was obtained 18 hr after the intraperitoneal injection of PAA. The mechanism of interferon production by PAA and PMAA is discussed. As described previously for Sindbis virus and endotoxin, the animals also became hyporeactive after injection of PAA.

Various nonviral substances have been reported to induce an interferon-like inhibitor both in tissue culture and in experimental animals. These include microorganisms, such as trachoma inclusion conjuctivitis (TRIC) agent (18), *Rickettsia tsutsugamushi* (H. E. Hopps et al., Bacteriol. Proc., p. 115, 1964), bacteria, like *Brucella abortus* (27), pleuropneumonia-like organisms (J. S. Youngner and W. S. Stinebring, *unpublished data*), and protozoa, like *Toxoplasma gondii* (8, 21). Some extracts of microorganisms likewise stimulate the production of interferon: bacterial endotoxins (11, 24) and extracts from *Candida albicans* (2), *Penicillium stoloniferum* (statolon) (15), and *P. funiculosum* (helenine) (22). The active compound of statolon and helenine, however, is ribonucleic acid of viral origin (1, 5, 14, 16). The antiviral activity of phytohemagglutinin, an extract from red kidney bean, *Phaseolus vulgaris*, might also be due to interferon (30). The interferon-inducing capacity of some synthetic products of defined composition, like cycloheximide (29) and pyran copolymers (17, 19), indicates that the production of interferon is not confined to complex biological derivatives. The study of synthetic anionic copolymers will add to the understanding of the physicochemical requirements for induction of interferon.

We were especially interested in polyacrylic and polymethacrylic acids, which are known from our previous report (4) to have striking antiviral properties in tissue culture. The possibility that both polyanions would act by stimulation of interferon was suggested though not proved. Since nonviral inducers have been reported to induce higher amounts of interferon in vivo than in vitro (25), the interferon-producing ability of PAA and PMAA was investigated in vivo.

MATERIALS AND METHODS

Female NMRI mice weighing about 20 to 25 g were injected intraperitoneally with 0.4 ml of the tested substance. Spleen homogenates were prepared as described elsewhere (3). The antiviral activity of sera and spleen homogenates was routinely determined with the plaque-inhibition technique by using secondary mouse embryo fibroblasts and vesicular stomatitis virus for challenge. After 24 hr, the neutral red staining was applied and the virus plaques were counted. A rapid interferon assay technique, based on the inhibition of vesicular stomatitis virus-cytopathogenicity in mouse embryo fibroblast culture tubes (3), was used for testing the diethylaminoethyl (DEAE)-fractionated samples.

Separation of interferon from synthetic polyanions in biological samples. Mouse or rat interferon was separated from synthetic polyanions on a column of DEAE-cellulose. As reported previously (23), rat tissue interferon is not retained on a column of DEAE-cellulose (*p*H 4.5) in 0.01 M sodium acetate buffer. Interferon was adsorbed by DEAE-cellulose (*p*H 7.0) in 0.01 M Sörensen phosphate buffer and eluted in 0.5 M (*p*H 7.0) phosphate buffer. Under the same conditions, PMAA and PAA were strongly retained on DEAE-

[1] "Aspirant" of the Belgian N.F.W.O.

cellulose. They were not eluted with 4 M acetic or formic acid, with 1 M sodium acetate buffer (*p*H 4.5), or with 1 M sodium chloride buffered with 0.1 M tris-(hydroxymethyl)aminomethane buffer (*p*H 8.2). These experiments showed that conditions sufficient to promote the elution of interferon did not remove the adsorbed polymer from the column.

To separate interferon from polyanions, the sample to be tested was dialyzed against 0.01 M sodium acetate (*p*H 4.5) and chromatographed on a small column of DEAE-cellulose previously equilibrated with the same buffer. Batch absorption was performed in centrifuge tubes containing approximately 1 ml of DEAE-cellulose equilibrated with 0.01 M sodium acetate buffer (*p*H 4.5). DEAE (0.68 meq/g; SS Serva, Heidelberg, Germany) was used in our experiments. Its capacity exceeds the amount of polymers that could be absorbed from our samples.

The concentration of the polymer was estimated turbidimetrically by precipitation at neutral *p*H with quaternary ammonium cetyltrimethyl ammonium bromide (Cetavlon; ICL, Wilmslow, England).

Substances tested. PAA, PMAA, and polystyrene sulfonate (sodium salt) were prepared as described previously (4).

Commercial samples of dextran sulfate 500 (Pharmacia, Uppsala, Sweden), polyvinyl sulfate (K-salt; Serva, Heidelberg, Germany), polyphloroglucinol phosphate (Leo Co. 137A; Hälsinborg, Sweden), heparin (RIT Co., Genval, Belgium), alginic acid (Light Co., Colnbrook, England), and macrodex (Pharmacia, Uppsala, Sweden) were also tested. Endotoxin was prepared from *Proteus rettgeri* according to the method of Roberts (20). A complex of polysaccharide was obtained by alkaline extraction of fish meal. Carboxylated dextran was prepared from macrodex by the method of Gilboe and Bock (9). The technique of Hjerten (10) was applied for the preparation of agaropectin. Once precipitated, the agaropectin was dissolved in 0.02 M HCl and separated from the contaminating Cetavlon by precipitation with 5 volumes of alcohol. A sulfated polysaccharide extracted from seaweeds and commercially available as "Ebimar" (Evans Medical Ltd., Speke, Liverpool, England) was used after dialysis against phosphate-buffered saline, at *p*H 7.4, to remove the superfluous aluminium hydroxide. The pyran (maleic anhydride divinyl ether) copolymer (NSC 46015-C; molecular weight, 17,000) was kindly supplied by T. C. Merigan (Palo Alto, Calif.).

RESULTS

Preliminary experiments showed that intraperitoneal and intravenous injections of PAA and PMAA were able to elicit significant antiviral activity in the serum of mice. Antiviral activity reached a peak titer after 18 hr, declined slowly, and disappeared completely after 48 hr. Therefore, blood samples were taken 18 hr after intraperitoneal injection of PAA, PMAA, or related polymers in our later tests.

Dose-response curves. Groups of five mice were injected intraperitoneally with increasing amounts of PAA or PMAA (Fig. 1) and the animals were bled 18 hr later. The antiviral activity of the pooled sera was assayed with the plaque-inhibition technique and expressed as interferon units. The serum activity induced by PMAA increased regularly with increasing amounts of compound, whereas the response to PAA reached its maximal effect from a 1.0-mg dose (Fig. 1). These dose-

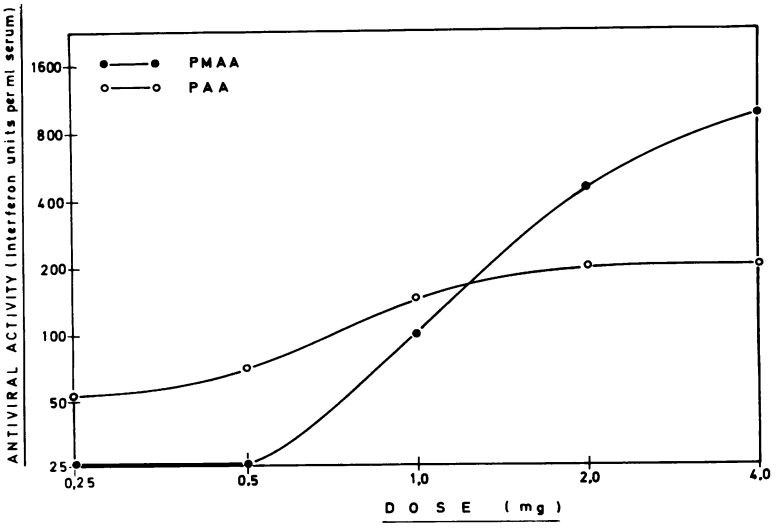

FIG. 1. *Dose-response curves. Antiviral activity in the serum 18 hr after intraperitoneal injection of either PAA or PMAA.*

TABLE 1. *Comparison of antiviral activity induced in serum 18 hr after intraperitoneal injection of PAA, PMAA, and other related polymers in mice*

Compound	Dose (mg/mouse)	Serum activity
PAA	4	1,250[a]
PMAA	4	925
"Ebimar"	2	525
Agaropectin	4	200
Alginic acid	20	155
Pyran copolymer	4	90
Dextran sulfate 500	4	66
Macrodex	24	60
Fish-meal polysaccharide	4	45
Endotoxin	0.8	<25
Dextran carboxylate	4	<25
Heparin	4	<25
Polyvinyl sulfate (K-salt)	4	<25
Polystyrene sulfonate	4	<25
Polyphloroglucinol phosphate	4	<25
Phosphate-buffered saline		<25

[a] Antiviral activity expressed in interferon units per ml.

response curves suggested that the acryl-polyanions would act in a somewhat different way.

Comparison of the antiviral activity induced in vivo by PAA, PMAA, and other related polymers. Groups of five mice were injected intraperitoneally with high doses of PAA, PMAA, and related polymers (Table 1). Blood samples were taken 18 hr later. The sera were assayed for antiviral activity with the plaque-inhibition method.

PAA, PMAA, and the sulfated polysaccharide extracted from seaweeds ("Ebimar") were found to be most active (Table 1). Agaropectin, alginic acid, and pyran copolymer were also effective. Other compounds failed to induce any antiviral activity in the given experimental conditions.

Characteristics of the antiviral factor induced by PAA and PMAA in vivo. Species specificity, destruction by proteolytic enzymes, thermolability, *p*H stability, and nondialyzability belong to the generally established criteria to which a viral inhibitor must conform if it is to be accepted as an interferon. These characteristics were explored for the antiviral activity induced in the serum 18 hr after the intraperitoneal injection of 4 mg of either PAA or PMAA per mouse, and compared to the properties of interferon appearing in the serum 8 hr after intravenous injection of Sindbis virus (Table 2). The action of viral inhibitor induced by PAA appeared to be consistent with the behavior of interferon. Conversely, the antiviral activity found after injection of PMAA did not meet the required interferon criteria: it persisted on heterologous cells, and was not destroyed either by trypsin or by heating.

The dose response of PMAA- and PAA-induced activity was then compared with the dose response of Sindbis virus interferon. When plaque reduction was plotted against interferon dilution on a logarithmic scale, an S-shaped curve with a linear intermediate portion was obtained (Fig. 2). The estimating equation, standard error, and coefficient of correlation of the regression lines were determined. The slopes of the activity curves of the PAA-induced inhibitor and the Sindbis-induced interferon were identical (Fig. 3 and 4). The slope of the PMAA curve, however, was quite different.

These results provided more evidence for the suggestion previously made that the activity of PAA and of PMAA were accomplished in different ways. The antiviral potency of PMAA may be due to a direct action of the compound as such, whereas PAA may act by induction of interferon.

Fractionation on DEAE-cellulose. A simple method for separating interferon from direct antiviral action was described above. Sera of mice injected intraperitoneally 18 hr before with 4 mg of either PAA or PMAA were incubated with DEAE-cellulose at *p*H 4.5 (0.01 M sodium acetate buffer) and centrifuged. The supernatant fluid was collected (fraction A: *p*H 4.5). The residue was taken up in 1.0 M NaCl containing 0.1 M tris(hydroxymethyl)aminomethane (*p*H 8.2), incubated, and again centrifuged. The superna-

TABLE 2. *Characteristics of antiviral activity induced in serum 18 hr after intraperitoneal injection of either PAA or PMAA in mice*

Characteristics	PAA	PMAA	Sindbis-induced interferon
Species specificity			
Activity in rat embryo fibroblasts	40[a]	690	135
Activity in mouse embryo fibroblasts	230	690	4,000
Sensitivity to trypsin			
1.25‰ at 36 C for 1 hr	50	920	<25
Control	500	850	615
Stability to heat			
56 C for 0.5 hr	<50	1,320	<50
Control	400	1,120	262
Dialyzability at *p*H 2			
4 C for 16 hr	780	1,120	ND[b]
Control	850	1,120	ND

[a] Antiviral activity expressed in interferon units per ml.

[b] Not done.

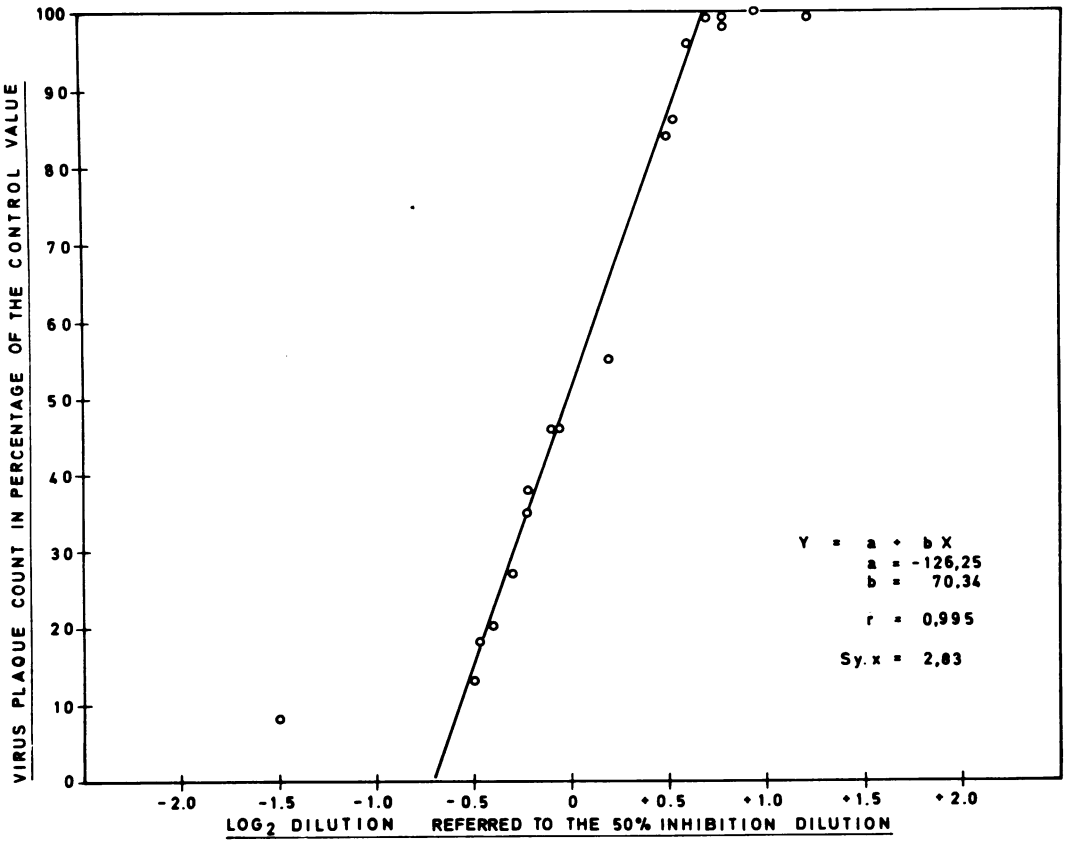

FIG. 2. *Dose-response relationship of PMAA-induced activity. Y = a + bx—estimating equation of the regression line; r—coefficient of correlation; Sy.x—standard error. Data refer to percentage of plaque reduction of subsequent dilutions of sera, either 18 hr after the intraperitoneal injection of PAA and PMAA or 8 hr after the intravenous injection of Sindbis virus.*

tant fluid was recovered (fraction B: *p*H 8.2). Both supernatant fractions were assayed by the cytopathic effect-inhibition method in mouse embryo fibroblast culture tubes with VSV as challenge virus (Table 3). The viral inhibitor appearing in the serum of animals injected with PAA showed little or no decrease after fractionation on DEAE-cellulose, whereas the inhibitor induced by PMAA disappeared almost completely after fractionation. The viral inhibitor, found in fractions A and B, might be identified with interferon, since the eluted fractions failed to induce any antiviral activity in heterologous cell systems (mouse embryo fibroblasts), lost their activity after exposure to trypsin (1.25% at 36 C for 1 hr) and heating (56 C for 0.5 hr), and remained active when dialyzed against *p*H 2.

It was concluded, therefore, that the whole antiviral activity of PAA resulted from interferon, whereas interferon played only a small role in the inhibitory potency of PMAA.

Influence of amidation on the interferon-inducing capacity of PAA. The interferon-inducing capacity of PAA may be a function of the presence of ionic charges on the polyelectrolyte molecule. To test this hypothesis, the interferon-stimulating properties of PAA were studied after partial or complete neutralization of the acidic functions with ammonia.

Polyacrylic acid-polyacrylamide copolymers, with varying proportions of the two components (Table 4), were intraperitoneally injected in groups of 5 mice (4 mg per mouse). Blood samples were taken 18 hr later and spleens were removed. Both sera and spleen homogenates were assayed for antiviral activity with the plaque overlay method. Only the pure PAA and, to a lesser degree, the copolymer provided with the highest amount of carboxyl groups (85%) were found effective (Table 4). The other copolymers and polyacrylamide itself were completely unable to induce activity. These data revealed the absolute

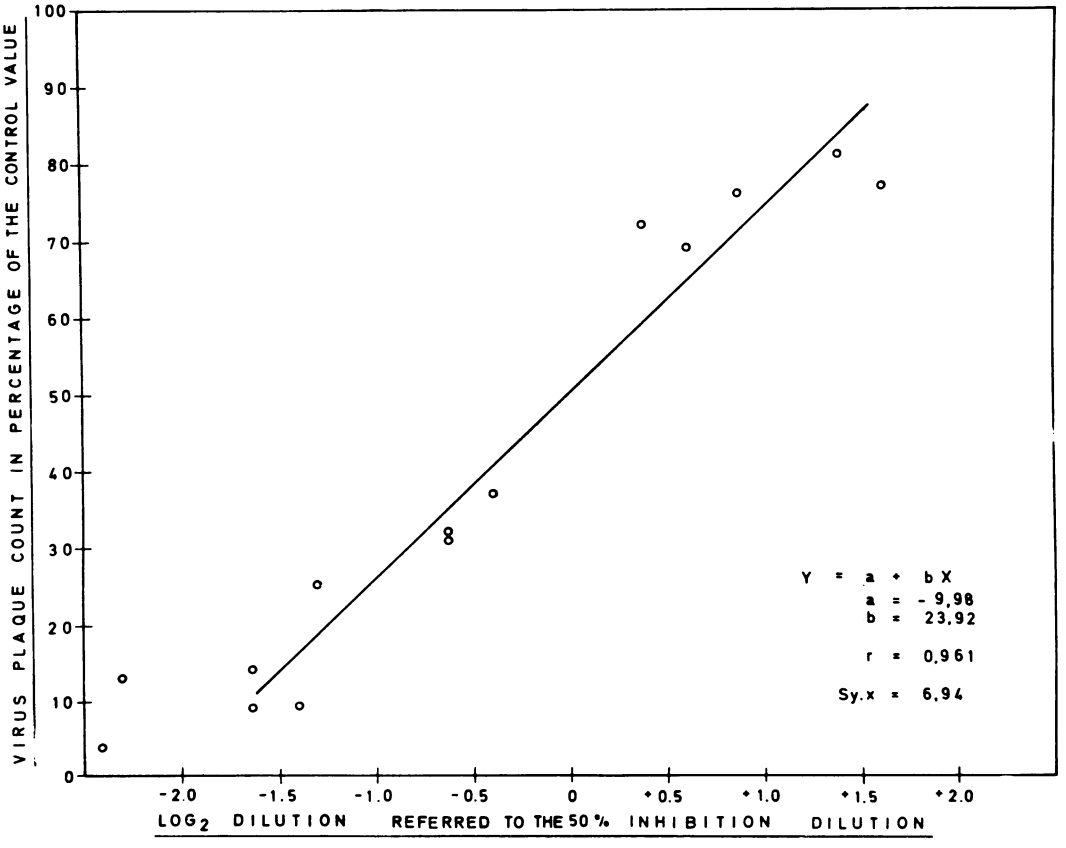

FIG. 3. *Dose-response relationship of Sindbis-induced interferon.*

necessity of free acidic groups on the polymer backbone for induction of interferon.

Hyporeactive state following administration of PAA. Rabbits, rats, or mice, injected with either virus or endotoxin and several hours later treated again with virus or endotoxin, showed a reduced interferon response when compared with controls not pretreated (3, 12, 13, 28). Hyporeactivity to the interferon-inducing effect of PAA was determined in similar experiments. Groups of five mice were given an intraperitoneal injection of either 0.5 mg or 2.0 mg of PAA. At different times, varying from 0 to 8 days after this injection, the animals received a second dose of 2.0 mg. Blood samples and spleens were taken 18 hr later and assayed for interferon with the plaque inhibition method. The interferon response to a second dose of PAA decreased. Hyporeactivity was dependent on both dose and time: maximal inhibition of interferon induction by a second dose was reached 8 days after the injection of a first dose of 2 mg. The inhibition was less pronounced at shorter time intervals and with 0.5 mg as the first dose (Fig. 5).

A striking parallelism between hyporeactivity and splenomegaly was observed: the reduction of interferon response was found proportional to an increase of spleen weight. Whether this peculiar parallelism implies any causal relationship remains an open question.

DISCUSSION

Striking antiviral activity appeared in sera of mice injected intraperitoneally with PAA and PMAA. These inhibitory effects resulted both from direct antiviral action affecting the virus-cell interaction and the viral ribonucleic acid metabolism (previous report) and from induction of interferon. The activity of PMAA was due mainly to direct antiviral action, whereas PAA acted primarily by stimulation of interferon production.

The exact mechanism of interferon induction is still unknown. The wide variety of interferon inducers (viruses, bacteria, protozoa, and biological extracts such as endotoxin, phytohemagglutinin, statolon, and helenine) do not reveal any common properties. Since synthetic anionic polymers [like maleic divinyl ether copolymer (17,

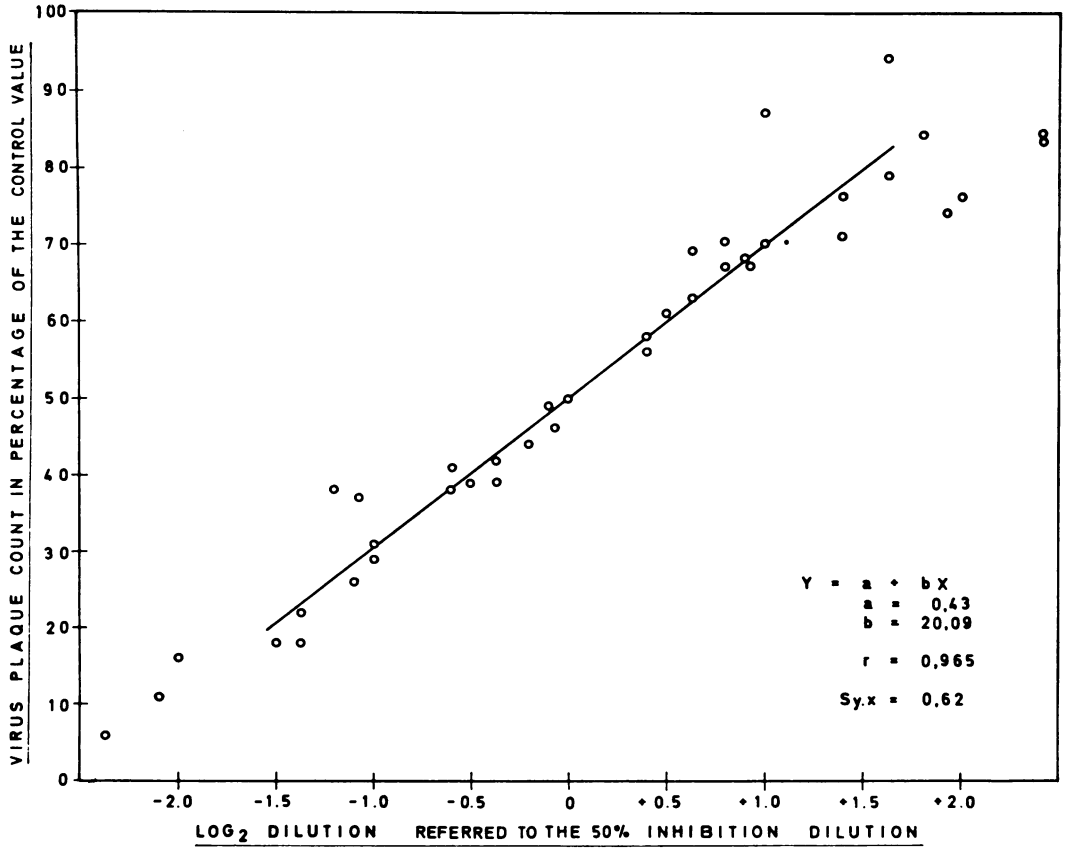

FIG. 4. *Dose-response relationship of PAA-induced activity.*

TABLE 3. *Fractionation on DEAE-cellulose of the antiviral activity[a] induced in the serum 18 hr after intraperitoneal injection of either PAA or PMAA in mice*

Sample	PAA	PMAA
Unfractionated serum.....	800	2,800
Fraction A (*p*H 4.5).....	640[b]	320
Fraction B (*p*H 8.15).....	120	0

[a] Expressed in interferon units per ml.

[b] Values obtained for fractions A and B must be identified with interferon, since they fulfilled the generally required (mouse) interferon criteria (species specificity, sensitivity to trypsin and heating, *p*H stability, and nondialyzability).

TABLE 4. *Influence of amidation on the interferon-inducing capacity of PAA*

Copolymer (per cent PAA/per cent polyacrylamide)	Interferon units[a]	
	Serum (unit/ml)	Spleen (unit/g)
100/0	3,200	2,920
85/15	710	420
58.5/41.5	<25	<250
23/77	<25	<250
0/100	<25	<250
Control	<25	<250

[a] Data refer to interferon concentrations in spleen and sera 18 hr after the intraperitoneal injection of the copolymer

19) and acrylic polymers] are found to produce high interferon titers, the question arises whether the polyanionic charge may play a significant part in the production of interferon. In any event, the polyanionic net charge appears to be absolutely required for the interferon-inducing ability. Amidation of the carboxyl groups of PAA to the extent of 40% results in a complete loss of induction capacity. Uncharged macromolecules (macrodex and polyacrylamide) are quite ineffective. The polyanionic structure is necessary but not effective. Polysulfate (dextran sulfate, polyvinyl sulfate, and heparin) and polyphosphate (polyphloroglucinol phosphate) polyelectrolytes fail to stimulate the production of interferon.

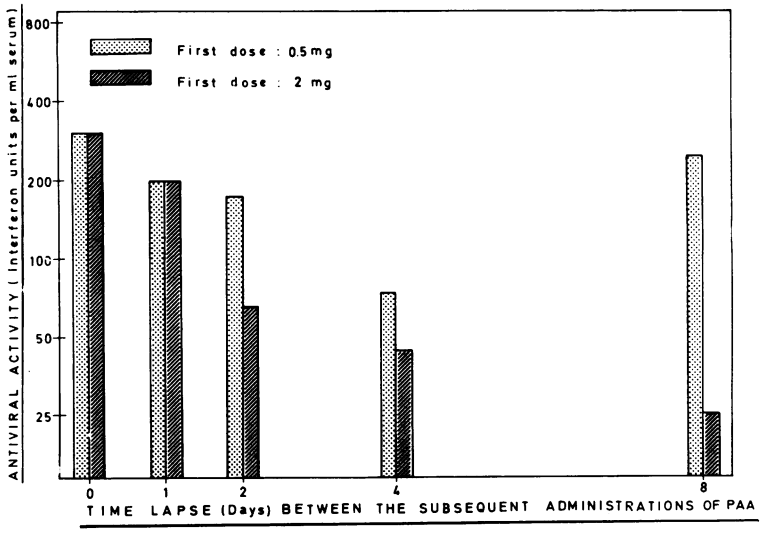

FIG. 5. *Hyporeactive state following administration of PAA. Serum interferon levels 18 hr after the intraperitoneal injection of the second dose (2 mg) of PAA.*

Agaropectin, alginic acid, and the commercial product "Ebimar" show little activity. Since their carboxyl groups are much more dispersed over the polymer backbone than for PAA and PMAA, the different charge distribution could account for the difference in interferon-inducing ability.

The capacity for inducing interferon by ribonucleic acids would depend on the double-strandedness of ribonucleic acid (6, 7, 16, 26). Whereas double-stranded ribonucleic acid obtained from extracts of *P. funiculosum* and from reovirus 3 virions and some synthetic double-stranded polynucleotides were found active in microgram amounts, other complexed polynucleotides of analogous structure failed to stimulate interferon production. Hence, we conceived the hypothesis that, more than multistrandedness, the steric configuration or physicochemical characteristics (charge-density and charge-distribution) of the anionic functions of the polymer are decisive for interferon formation. Apparently, polyacrylic polymers, pyran copolymers, and some multistranded polynucleotides do conform with the specific configuration, charge-density, and charge-distribution required for activity. The exact nature of these specific conditions, however, needs further study.

The injection of PAA resulted in a progressively increasing hyporeactive state during which the interferon response to a second dose was reduced. Many possibilities may be considered to explain these tolerance phenomena. It could be attributed to an exhaustion of the interferon store, though this possibility does not account for the progressive increase of hyporeactivity. It is more likely that PAA impairs some important cells or metabolic reactions involved in the production of interferon.

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