Pure & Appl. Chem., Vol. 46, pp. 103–113. Pergamon Press, 1976. Printed in Great Britain.

BIOLOGICALLY ACTIVE SYNTHETIC POLYMERS†

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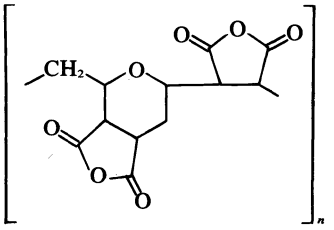
Abstract—The 1:2 divinyl ether-maleic anhydride cyclic alternating copolymer (DIVEMA) shows a wide variety of biological activities. It has antitumor activity; it induces the formation of interferon; it has antiviral, antibacterial, and



antifungal activity; it is an anticoagulant and an anti-inflammatory agent. DIVEMA is an immunopotentiator; it increases the rate of phagocytosis, it activates macrophages selectivily, and it inhibits RNA-dependent DNA polymerase.

The effect of molecular weight and molecular weight distribution on the biological activity and toxicity of DIVEMA has been investigated and will be discussed.

It seems most fitting, in light of Prof. Aharon Katzir-Katchalsky's deep interest in the field, to limit this discussion of biologically active synthetic polymers to those which are polyelectrolytes.

The use of synthetic polymers in medicine is growing rapidly. But in a certain sense most polymers are not used because of their biological activity. Initially they were utilized as structural materials, first as replacements for metals, but subsequently for their own favorable properties—ease of fabrication, inertness to body fluids, low cost, and ready availability. Today many different materials are in use, from silicone rubber heart valves to polyethylene hip joints, from polyester arteries to acrylic teeth. However, these materials are used, if anything, for their biological inertness and not for their biological activity. In a similar sense, dextran and polyvinylpyrrolidone were used as plasma extenders because of their effect on osmotic pressure, and not because of any special biological activity. A considerable literature exists on the use of polymers as carries for a variety of drugs, with the pharmacon physically incorporated or covalently bound to the polymer.[1-3] In fact, a disk of polymer containing pilocarpine has recently become commercially available for the treatment of glaucoma, and an IUD containing a year's supply of contraceptive steroid is about to be marketed.

There are a large number of naturally occurring anionic polyelectrolytes, some of which are shown in Table 1.

Table 1. Naturally occurring anionic polyelectrolytes

Proteins	Plant gums:
DNA	Pectin
RNA	Gum Arabic
Hyaluronic acid	Gum Tragacanth
Chondroitin sulfate	Agar
Heparin	Carrageenan
	Alginic acid

The importance of proteins, DNA, and RNA needs no additional comment. Hyaluronic acid is the jelly-like matrix which is the cement substance of the tissues; It is a polysaccharide which yields D-glucuronic acid on hydrolysis. Chondroitin sulfate is widely distributed in animal tissue (cartilage, tendons, skin); it is a polysaccharide containing both carboxyl and sulfate groups. Heparin, as its name indicates, is found in the liver. It too is a polysaccharide containing carboxyl, sulfate, and sulfamic acid groups; it is used clinically as an anticoagulant. Finally there are the plant gums, which are used broadly in the food and chemical industries as thickening agents.[4] They too are polysaccharides which owe their anionic character to free carboxyl groups, although agar and carrageenan contain sulfate groups as well. Materials of animal origin generally seem to contain acylated amino groups in addition, whereas the plant materials do not. In comparison, cationic polymers are relatively rare and are usually polypeptides, e.g. protamines and histones.

It is not surprising that interest, which centered originally on naturally occurring polyelectrolytes, has expanded recently to include synthetic polyelectrolytes as well. Some of the earlier work involved polymers of sodium ethylenesulfonate, whose polymerization was first reported in 1954.[5] Regelson and Holland[6] found a wide spectrum of antitumor activity, in mice, for the sodium salt (Table 2).

Table 2. Sodium polyethylenesulfonate tumor inhibition in mice

Adenocarcinoma 775	L5178 lymphatic leukemia
L1210 lymphoid leukemia	Ehrlich (ascites)
Krebs 2 carcinoma (ascites)	Sarcoma 180

Unfortunately, the activity in humans appeared to be much lower and the polymer was too toxic for clinical use.[7] Polymeric sulfates and sulfonates all appear to have heparin-like properties, and for a time Farbwerke Hoechst marketed sodium polyethylenesulfonate under the trade name Pergalen for topical application as a blood

†Hercules Research Center Contribution No. 1674.

Table 3. Antitumor activity of divinyl ether-maleic anhydride copolymer (NSC46015)

	Antitumor activity	Max. tol. dose/ min. eff. dose (mg/kg)	Therapeutic index
Adenocarcinoma 755	+	40/10	4
Lewis lung carcinoma	+	32/4	8
Friend leukemia virus	+	80/10	8
Dunning ascites leukemia	+	60/7.5	8

anticoagulant.[8,9] Although the product is apparently no longer offered, this was probably one of the earliest clinical applications of a synthetic polyelectrolyte.

A number of carboxylic acid polymers—poly(acrylic acid), poly(methacrylic acid), and ethylene-maleic anhydride copolymer—were found to have antineoplastic acitivity similar to that of sodium polyethylenesulfonate,[10] but here too toxicity precluded clinical evaluation.[11]

Because of our general interest in water-soluble polymers, we had prepared the 1:2 divinyl ether-maleic anhydride copolymer originally reported by Butler.[12] Submission of a number of samples to the National Cancer Institute showed that the hydrolyzed and neutralized polymers also had antitumor activity, but this was coupled with a much lower toxicity than had been observed with previously investigated anionic polymers. Preliminary results with one sample, designated NSC 46015, are shown in Table 3.[13]

Table 4[14] shows its activity against Lewis lung carcinoma, a slow-growing, solid tumor which is very difficult to control.[15,16] The activity is comparable to that of cyclophosphamide, a widely used alkylating agent, whereas 6-mercaptopurine, an antimetabolite, is essentially devoid of activity. The results are given as the mean survival time of the treated animals divided by that of the control.

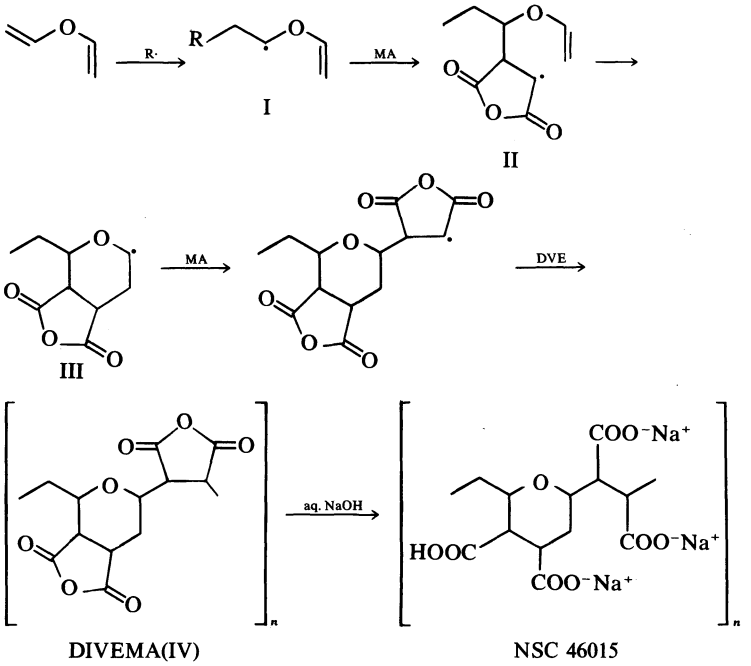
Because of the copolymer's rather broad spectrum of activity and its acceptable therapeutic index, the National Cancer Institute investigated it quite intensively, and it

Table 4. Activity of NSC 46015 against Lewis lung carcinoma

Drug	Dose (mg/kg/injection)	T/C,% (mean survival time)
Cyclophosphamide	25	> 135, > 136
6-Mercaptopurine	40	–, 100
	20	113, 108
NSC 46015	50	> 141, > 152
	25	133, > 159

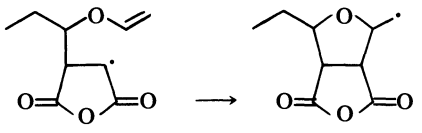
was eventually approved for experimental clinical evaluation.

Divinyl ether (DVE) and maleic anhydride (MA) copolymerize in a 1:2 ratio with a radical catalyst, presumably in the following fashion.[12] We use the simple acronym, DIVEMA, for divinyl ether-maleic anhydride copolymer. However, based on the assumption of a tetrahydropyran ring in the structure, it almost immediately became known as Pyran Copolymer, and in fact, it is indexed as such in the medical literature. As shown here, a radical attacks one of the double bonds of divinyl ether to give the secondary radical (I). Since vinyl ethers copolymerize well with maleic anhydride, the next step would be expected to be the addition of I to maleic anhydride to give radical II. If this radical then attacks the β-carbon of the remaining vinyl ether group, a six-membered ring would be formed, and the resulting radical (III) would again react with maleic anhydride, giving the

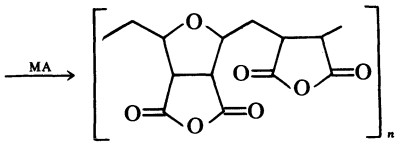


1:2 copolymer (IV). Potentiometric titration has shown that at pH 7 approximately three of the four carboxyl groups are neutralized, as illustrated in NSC 46015 (no significance should be attached to the position of the carboxylic acid, although titration suggests that one carboxyl group is different from the other three).

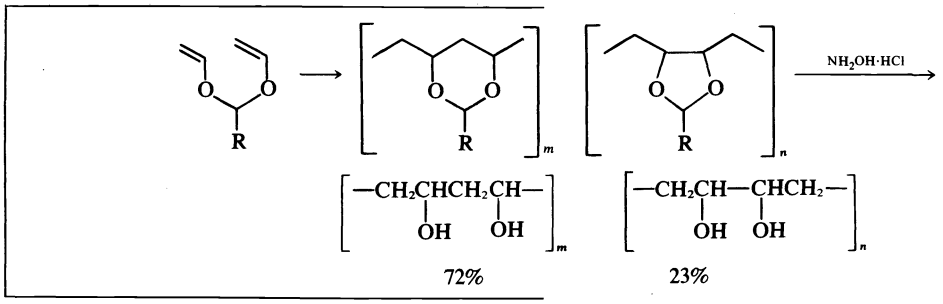
Although this structure has been widely accepted,[16a] there is no convincing evidence for it. There is little doubt that a 1:2 copolymer is formed, although good combustion analyses are difficult to obtain. Analysis shows only a trace of unreacted vinyl ether. However, there are a number of alternative structures which cannot be eliminated from consideration. Thus, if the succinate radical in II were to add to the α-rather than to the β-vinylic carbon, a polymer containing tetrahydrofuran rings (VI) would be formed instead of IV.



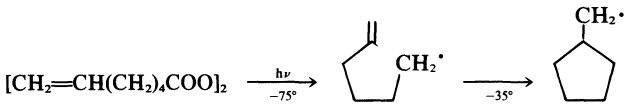
II V



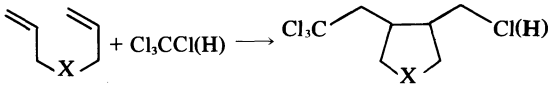
VI



Although at first sight it seems unreasonable to form a primary radical (V) in preference to the more stable secondary radical (III), a considerable body of evidence has been accumulating that the formation of five-membered rings is preferred over six, i.e. the reactions are under kinetic rather than thermodynamic control. Thus, Kochi and Krusic[17] found that the 5-hexenyl radical, formed by low-temperature photolysis of 6-heptenoyl peroxide in the cavity of an ESR spectrometer, was converted exclusively into cyclopentylmethyl radical.



Brace[18] studied the telomerization of a number of diallyl compounds—1,6-heptadiene, ethyl diallylacetate, N-methyldiallylamine, diallyl ether, etc.—with a number of telogens—carbon tetrachloride, bromotrichloromethane, perfluoroalkyl iodides—and in no case did he find evidence for the formation of other than five-membered rings. Aso and coworkers[19] telomerized chloroform with diallyl ether and with divinyl ether and in both cases obtained compounds which contained a methyl group; methyl groups can be formed only if a five-membered ring is obtained.

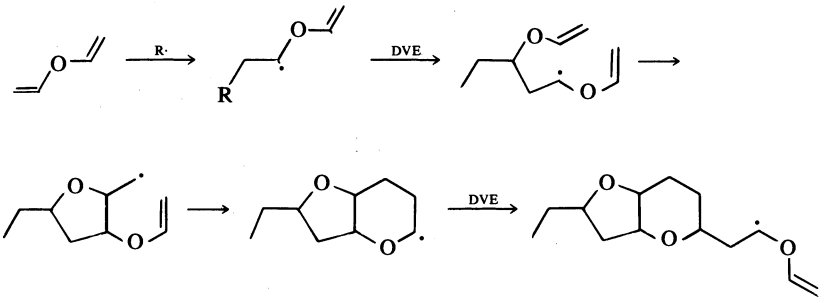


X = CH_2, $CHCO_2Et$, NMe, N—CN, O, etc.

Although there is some doubt that telomerization and polymerization follow the same paths,[19,20] there are numerous examples of cyclopolymerization in which five-membered rings are formed predominantly or exclusively. Thus, Solomon,[21] using a combination of ESR, NMR, and chemical analysis, showed that methyldiallylamine added radicals and polymerized to form predominantly, if not exclusively, five-membered rings. In other cases mixtures of five- and six-membered ring compounds are formed. *N*-methyldimethallylamine gave a polymer containing both rings, the percentage of six-membered ring increasing with increasing temperature. This is in agreement with Julia and Maumy,[22] who found that more stable radicals give greater amounts of six-membered ring in radical cyclization reactions. Arbuzova and coworkers[23] polymerized divinyl acetals, hydrolyzed the polymers to the corresponding gylcols, and determined 1,2-glycol content by periodate cleavage. The ratio of five-membered to six-membered ring content was about 1:3; this would appear to be the most clear-cut evidence that the early assumptions that cyclopolymerization always yielded six-membered rings is incorrect.

Thus far we have had little success in proving the structure of DIVEMA. Both the proton and ^{13}C magnetic resonance spectra are too complex for analysis at the present time. The very complexity might suggest the presence of both five- and six-membered rings, but the number of chiral centers in both IV and VI would be enough to account for the large number of peaks.

Still another complication arises from the possibility of chain-branching in the polymer. Although alkyl vinyl ethers do not homopolymerize with a free-radical catalyst, divinyl ether does. At low conversions a soluble polymer can be obtained, which contains only about 20% of the expected unsaturation, so that undoubtedly cyclic polymers are being formed;[24] here too, there is little direct evidence as to ring size, and polymers with both five- and six-membered rings may be formed, with vinyl ether groups pendant from the ring or the chain.[25]



According to Butler,[26] the rate of divinyl ether homopolymerization is approximately a tenth that of its copolymerization with maleic anhydride, so the probability is quite high that some structures of this type would be formed during the polymerization. Since maleic anhydride copolymerizes well with vinyl ethers, the dangling vinyl ether groups would be branch points, leading to long-chain branching in the copolymer, and the degree of unsaturation in the final polymer should be low, as found.

One factor which might affect the structure of the polymer is formation of a 1:1 charge-transfer complex from divinyl ether and maleic anhydride as reported by Butler,[27] so that the actual polymerization might be an alternating copolymerization between this complex and maleic anhydride. It is quite conceivable, of course, that this copolymerization would lead to an unbranched polymer with exclusively six-membered rings. However, the charge-transfer complex is a very weak one, and in polar solvents its concentration would be extremely low, if it would be present at all. In summary, divinyl ether forms a 1:2 copolymer with maleic anhydride in a radical polymerization. The copolymer is almost completely saturated, so cyclic structures must be present; whether the polymer contains five-membered rings, six-membered rings, or a mixture of the two is not known. Whether the copolymer is linear or branched is also unknown, although what little evidence is available suggests that it is predominantly linear.

DIVEMA possesses a broad spectrum of biological activity (Table 5). The induction of interferon, the protein which appears to be the body's first line of defense against viral infection, was first suggested by Regelson[28] to account for the antitumor and antiviral activity of DIVEMA. Interferon induction in mice was demonstrated by Merigan[29] (Table 6).

Table 5. Biological activity of DIVEMA

Antitumor	Anticoagulant
Interferon inducer	Antiarthritic
Antiviral	Elimination of Pu
Antibacterial	Inhibits reverse transcriptase
Antifungal	Activates macrophages

According to Merigan, above a dose level of about 75 mg/kg there is no effect of dosage on interferon formation. Regelson found little effect of increasing molecular weight of the copolymer on activity, whereas Merigan found a decrease. Actually, the activity of DIVEMA is not particularly outstanding, but it was one of the first, if not the first, well-defined material to show interferon induction. Its activity as an inducer of interferon has been confirmed in humans.[30]

The reports of interferon induction by DIVEMA led to its evaluation against a number of viruses, and it has been shown to have a broad spectrum of activity, including

Table 6. Induction of interferon in mice by DIVEMA

Sample	RSV (0.1% in 1 M-NaOH)	Dose (mg/kg)	Interferon units R[28]	Interferon units M[29]
Control			< 20	
NSC 46015	0.22	25		20
		75		230
		125	187	210
		250		220
XA124-177	0.31	125	159	185
NSC 68987	0.68	125	246	97
NSC 68988	1.5	125	205	78

activity against a number of cancer-inducing viruses: Friend leukemia,[28,31,32] Rauscher leukemia,[32] Moloney sarcoma,[31] vesicular stomatitis,[33] Mengo,[34] MM,[35] vaccinia,[36] encephalomyocarditis,[37] and foot-and-mouth disease.[38-40] Figure 1 shows the protection afforded mice against two different dilutions of murine sarcoma virus (Moloney)-Plasma Variant by five daily injections (25 mg/kg) of DIVEMA prior to i.p. injection of virus.[31] At both virus dilutions most of the control mice were dead within 60 days. At a hundred-fold dilution of virus only 40% of the treated mice had died within 140 days, whereas at a thousand-fold virus dilution the mice were completely protected by DIVEMA. One thing which should be noted is that this is a prophylactic treatment, that is, the DIVEMA must be given before the viral challenge. This has turned out to be generally true of DIVEMA and of other related materials.

Figure 2 shows some very exciting results by Campbell

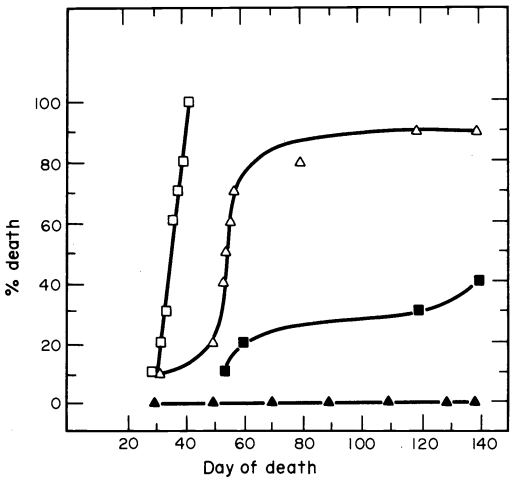


Fig. 1. Protection of mice against Moloney sarcoma virus—plasma variant by DIVEMA. Virus dilution 10^{-2}, □ control, ■ treated; 10^{-3}, △ control, ▲ treated. Reproduced by kind permission of S. Karger AG, Basel from the paper by M. A. Chirigas, *Comparative Leukemia Res.* 289 (1969).

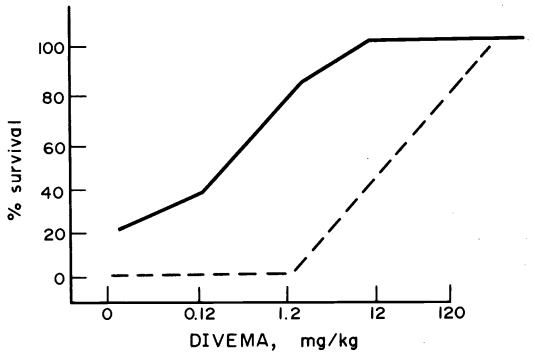


Fig. 2. Influence of DIVEMA on resistance of 7-day-old mice mediated by foot-and-mouth disease virus vaccine. ---- DIVEMA alone; ——DIVEMA + vaccine. Reproduced by kind permission of the American Society for Microbiology from the paper by C. H. Campbell and J. Y. Richmond, *Infection and Immunity*, 7, 202 (1973).

and Richmond,[39] who found a synergistic effect between DIVEMA and foot-and-mouth-disease vaccine in mice. Seven-day-old mice are not protected by vaccine; a 1.2 mg dose of DIVEMA plus vaccine protected 80% of the mice, whereas at this very low dose level DIVEMA alone showed no appreciable activity. Unfortunately, the activity shown by DIVEMA alone against foot-and-mouth disease in mice and guinea pigs has not carried over to cattle and pigs; it would appear that the dosages used were too high, and toxic side effects were observed.[40,41] Apparently, vaccine beneficiation has not been investigated in large animals.

DIVEMA has been shown to have antibacterial activity against both gram-positive (*Listeria monocytogenes*,[42] *Diplococcus pneumoniae*,[43,44] *Staphylococcus aureus*[45]) and gram-negative (*Klebsiella pneumoniae*,[46] *Pasteurella tularensis*[44]) organisms. For example, Remington and Merigan[42] found that mice treated with DIVEMA became resistant to *L. monocytogenes* after about four days, and remained resistant for as long as two months. Here, too, the treatment is prophylactic, as if some agent must be built up before immunity is established.

DIVEMA has also shown antifungal activity.[43] *Cryptococcus neoformans* is a lethal yeast which attacks the lungs and brain of mice. Thus, mice given two treatments of DIVEMA (25 mg/kg i.v.), either a week or several days before challenge had a mean survival time of greater than 90 days; untreated controls had a survival time of 19 days. The treated mice appeared perfectly healthy, in spite of the fact that the presence of *C. neoformans* could be demonstrated.

Figure 3 shows the inhibition of adjuvant disease in rats by DIVEMA.[47] Adjuvant disease is believed to be due to a hypersensitivity reaction to mycobacterial antigens, and it

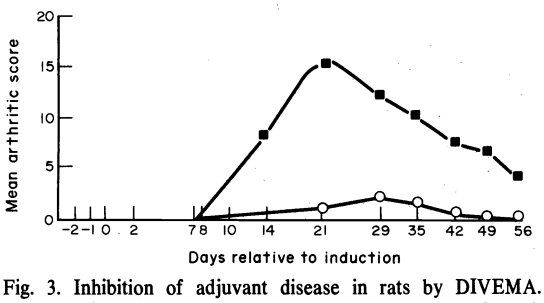


Fig. 3. Inhibition of adjuvant disease in rats by DIVEMA. Adjuvant injection at day 0, DIVEMA at days −1, +7, +14. ■Control (Hanks' balanced salt solution), ○ DIVEMA.

is considered of importance because of the similarity of the animal disease to rheumatoid arthritis. Since this is a delayed reaction, rats were protected if DIVEMA was injected one day before or seven days after the adjuvant, but not fourteen days after.

Several reports on the anticoagulant action of DIVEMA have appeared recently.[48,49] According to Shamash and Alexander,[48] fibrinogen clotting by thrombin is extremely sensitive to low concentrations of DIVEMA, about fourfold more than to heparin. Several clinical observations have been reported that DIVEMA, although a potent anticoagulant, does not cause bleeding.[50,51]

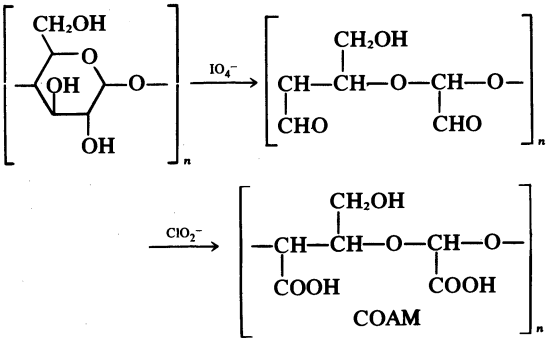
Scientists at the Argonne National Laboratory have reported that DIVEMA, in combination with a chelating agent, diethylenetriaminepentaacetic acid, is more active in removing polymeric plutonium from the liver than a number of other materials investigated.[52]

A number of other anionic polymers show similar biological activity to DIVEMA; some of these are listed in Table 7.

Table 7. Synthetic polyelectrolytes with biological activity

Sodium polyethylenesulfonate[6]
Poly(acrylic acid)[10]
Poly(methacrylic acid)[10]
Ethylene-maleic anhydride copolymer[10]
Acrylic acid-maleic acid copolymer[53]
Acrylic acid-itaconic acid copolymer[53]
Polyinosinic-polycytidylic acid (Poly rI·Poly rC)[54]
Chlorite-oxidized oxyamylose (COAM)[55,56]

Much of the work in this field, especially the medical aspects, has been well summarized by Regelson.[11,57] The first three carboxylic acids appear to have a broad spectrum of activity, but, as already mentioned, they were too toxic for clinical evaluation. Only limited reports are available on the acrylic acid copolymers. COAM is the most active of a group of polysaccharide derivatives prepared by cleaving amylose with periodate and oxidizing the resulting aldehyde groups to carboxyl groups with sodium chlorite.



COAM has antiviral activity and appears to be relatively non-toxic, but clinical evaluation has not been reported.

The most intensively studied material in Table 7 is the synthetic double-stranded RNA, poly I·poly C. It is one of the most potent interferon inducers known, and it possesses a broad spectrum of activity. The literature is too voluminous to be discussed here, but several good reviews exist.[58,59] Thus far its clinical activity has been disappointing, especially against cancer.[59a]

How does one explain the bewildering variety of activities shown by DIVEMA and related materials? For a

Table 8. Effect of DIVEMA on Rauscher virus leukemia in normal and immunosuppressed C_3H mice

Thymectomy (day −10)	ALS (days −8 and −6)	DIVEMA (days −5 to −1)	Rauscher virus (day 0)	Mean spleen wt, g(day 42)	Reduction in virus titer (logs)
+	+	+	+	0.151 ± 0.011	>2
+	+	−	+	0.416 ± 0.078	0
−	−	−	+	0.349 ± 0.022	0
−	−	+	+	0.176 ± 0.009	>2

considerable time it was thought that the induction of interferon was responsible for the prophylactic action of DIVEMA, since the interferon level must be built up before the infection for it to exert a protective action. However, the prolonged action of DIVEMA argues against this, since it protects long after any circulating interferon can be found in the blood stream. Thus, Merigan and Finkelstein[34] found mice to be resistant to Mengo virus 60 days after a single injection of DIVEMA, whereas no measurable amount of interferon could be found after 4–6 days; its long-lasting activity against *L. monocytogenes*[42] and *C. neoformans*[43] has already been mentioned.

It seemed to be a logical alternative that DIVEMA, and other related materials, affects the immune response of the animal, and considerable work has been done to investigate this possibility. Regelson and coworkers[60] studied the interaction of DIVEMA with the reticuloendothelial system (RES) by investigating the effect of DIVEMA on phagocytosis in mice. Normally, foreign bodies are removed from the blood stream by a clean first-order reaction, so that one need simply determine the half-life. It is quite obvious from the results shown in Fig. 4 that the effect of DIVEMA is time-dependent. Thus, the half-life for an untreated mouse remained fairly constant at about 13.5 min for a number of foreign bodies. When colloidal carbon was injected two days after DIVEMA, the half-life doubled, i.e. the rate of phagocytosis decreased. After seven days, however, the half-life had decreased to 4 min, showing a marked acceleration compared to the control. Similar results were obtained with a lipid emulsion and with sheep red blood cells. Since the RES plays a defensive role in inflammation and immunity, its stimulation could explain the generalized activity of DIVEMA.

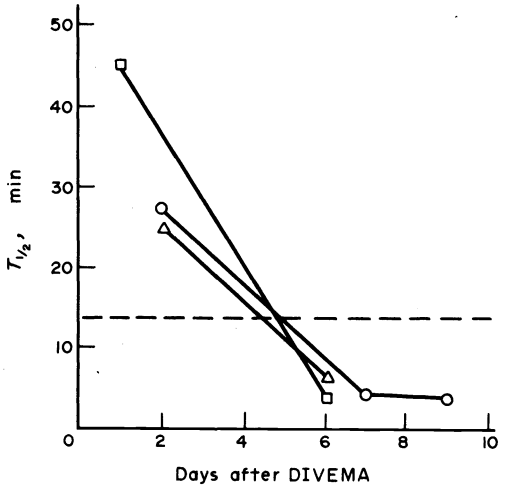


Fig. 4. Rate of phagocytosis in mice as a function of time after DIVEMA administration. ---- control (no DIVEMA); ○ colloidal carbon; △ lipid emulsion; □ ^{51}Cr sRBC.

In contrast to this, Hirsch and co-workers[61] showed the effect of DIVEMA on Rauscher virus leukemia in both normal and immunosuppressed mice (Table 8). Mice in the first two groups were severely immunosuppressed by being thymectomized and then treated with antilymphocyte serum. The next two sets were normal mice. In both normal and immunosuppressed mice, DIVEMA prevented the development of Rauscher virus-induced leukemia; the spleen weights were only slightly larger than those of untreated controls and no evidence of leukemia was found on histological examination of the spleens. As can be seen, in both normal and immunosuppressed mice DIVEMA caused a dramatic reduction in the virus titer. These results suggest that the action of DIVEMA was not a result of stimulating host immune response in this test.

These results, which have been confirmed in AKR mice,[62] are of considerable importance, even if they do not shed a great deal of light on the mode of action of DIVEMA. Since immunosuppressed patients "have a markedly increased incidence of both viral infections and cancer",[62] the fact that DIVEMA is active under these circumstances may be of major importance.

Still another mode of action of DIVEMA has been reported by Papas, Pry, and Chirigos,[63] who found it to be a potent inhibitor of viral RNA-dependent DNA polymerase (reverse transcriptase); examples are shown in Table 9. Bacterial DNA polymerases were activated under the same conditions. Activity appeared to increase with both increasing concentrations and increasing molecular weights of the DIVEMA samples.

Table 9. The effect of DIVEMA on RNA-dependent DNA polymerase

Viral enzyme	Activity: Without DIVEMA	Activity: DIVEMA (100 μg/ml)	Inhibition (%)
Avian myeloblastosis	500	40	92
Rauscher leukemia	105	45	57
Reptilian C-type	129	21	84
Pig kidney C-type	222	81	63

Still another instance of stimulation of host immunity was reported by Braun *et al.*,[64] who found DIVEMA to be a potent stimulator of macrophage activity. Kaplan, Morahan, and Regelson[65] showed that peritoneal macrophages taken from DIVEMA-inoculated mice were cytotoxic to B16 melanoma and Lewis lung carcinoma cells, *in vitro*, much more than to normal cells. The effect persisted with immunosuppressed mice, which could ac-

count for the protection afforded immunosuppressed mice by DIVEMA in the work of Hirsch *et al.*[61,62]

The use of DIVEMA as an adjuvant to chemotherapy by Chirigos and coworkers[66] has led to dramatic results. A Moloney lymphoid leukemia (MCAS-10) was injected into mice and allowed to grow. Then the mice were put into remission with 1,3 - bis(2 - chloroethyl) - 1 - nitrosourea (BCNU), a highly active alkylating agent. With no further treatment they relapsed, and only a third survived for 60 days. One treatment with DIVEMA while the mice were still in remission led to 92% survival, and additional treatments were only marginally more effective (Table 10).

Table 10. Chemoimmunostimulation therapy against MCAS-10

MCAS-10 (day 0)	BCNU (30 mg/kg)	DIVEMA (20 mg/kg)	Survivers, % (day 60)
1×10^4 cells			0
1×10^4 cells	day 10		33
1×10^4 cells	day 10	day 13	92
1×10^4 cells	day 10	days 13, 14, 15, 16, 17	83
1×10^4 cells	day 10	days 13, 15, 17, 19, 21	100

Other reagents showed a similar effect—*Bacillus Calmette-Guerin* (BCG), *Corynebacterium granulosum*, tilorone hydrochloride, and Levamisole. Chirigos considered all these materials to be nonspecific immunostimulators.

One interesting fact which must be considered in explaining the mode of action of anionic polyelectrolytes is the growing body of literature on the activity of these materials as plant virucides, a number having been reported to decrease the infectivity of tobacco mosaic virus.[67–70] The mechanism of action is unknown, although Kassanis *et al.*[71] claim to have isolated a number of proteins from treated plants which are not present in untreated, healthy plants. Whether or not there is the equivalent of interferon in plants is a matter of conjecture.

Unfortunately, DIVEMA, like all synthetic anionic polymers, shows a number of toxic side effects—pyrogenicity, thromobocytopenia, inhibition of microsomal enzymes, sensitization to endotoxin, liver damage, organomegaly, and depression of the reticuloendothelial system.[72] Although no one of these side effects is in itself sufficiently serious to preclude the use of DIVEMA, the overall combination is not very encouraging for long-term clinical use. This, plus several other observations, led us to a more thorough investigation of the nature of DIVEMA. First, we found that acute toxicity increases with increasing molecular weight (Fig. 5). Second, in order to determine the metabolic fate of DIVEMA, samples had been prepared with ^{14}C-labeled maleic anhydride.[73] Although the material was excreted

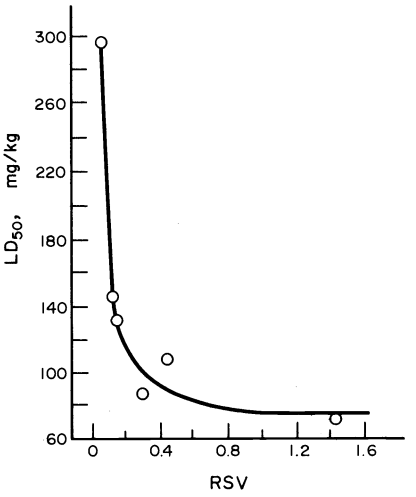


Fig. 5. Acute toxicity in mice of DIVEMA as a function of molecular weight. RSV (0·1% in 1 M NaOH), LD_{50} (mg/kg i.v.).

fairly rapidly, about 70% being lost in 5 weeks, a portion was excreted very slowly, some of it remaining even after 9 months. Third was the report of a biphasic response of the RES towards DIVEMA, the initial decrease of activity being followed by a considerable increase after several days.[60] Combining these observations, we hypothesized that the copolymer might consist of two materials, one toxic, very slowly metabolized, and responsible for the RES depression, and the other less toxic, rapidly metabolized, and responsible for the RES stimulation. We assumed that the two materials would differ only in molecular weight and that the high molecular weight portion was the undesirable material.

The initial copolymer approved for clinical investigation (NSC 46015) was prepared by a peroxide-catalyzed polymerization in benzene, using carbon tetrachloride as a chain-transfer agent to control the molecular weight, similar to the procedure reported subsequently by Butler.[12a] This is a slurry process; the polymer is insoluble in the solvent, and precipitates as it forms. This would be expected to lead to a polymer with a broad molecular weight distribution.

In order to characterize the polymers, attempts were made to carry out gel permeation chromatography (GPC) on Styragel columns of solutions of the anhydride in organic solvents; these were unsuccessful, presumably because of partial hydrolysis of the anhydride groups. We were similarly unsuccessful in attempts to fractionate solutions of the free acid in aqueous salt solution by GPC. Similar problems were encountered by Butler and Wu.[74] The polymers were therefore converted into their methyl esters by first refluxing them in methanol and then treating them with diazomethane; several diazomethane treatments were necessary to obtain complete esterification. These esters fractionated smoothly on a Styragel column using tetrahydrofuran as solvent. A comparison of the molecular weights of the anhydride and the methyl ester both by membrane osmometry and by light scattering demonstrated that no appreciable degradation had occurred during the esterification (Table 11).†

After it was shown that the methyl esters behaved normally, a large sample was separated on a preparative GPC column to obtain sufficient material for further investigation. These samples were then used for calibration purposes. The intrinsic viscosities and weight average

†We encountered none of the difficulties reported subsequently by Butler and Wu.[74] Analysis showed that reaction with methanol required 16–18 hr reflux for completion, and that treatment with diazomethane in a solvent for the methyl ester was necessary for complete esterification. It is conceivable that Butler and Wu's problems arose because of the presence of small amounts of free carboxyl groups, since they esterified with diazomethane in methanol, which is a nonsolvent for the ester. We followed the methanol treatment with one in tetrahydrofuran, which is a solvent for the partially esterified polymer.

Table 11. Molecular weights of DIVEMA and DIVEMA methyl ester†

	$\bar{M}_n \times 10^{-4}$	$\bar{M}_w \times 10^{-5}$	$\bar{M}_w/\bar{M}_n$
Anhydride	3.75	3.39	9.04
Methyl ester	4.56	4.20	9.21
Methyl ester (calc)‡	4.88	4.42	

†$\bar{M}_n$ by membrane osmometry, $\bar{M}_w$ by light scattering, both in tetrahydrofuran.

‡Based on the anhydride being 25% hydrolyzed before esterification.

molecular weights by light scattering of these fractions were determined in tetrahydrofuran. They were then analyzed on an analytical GPC column and compared with polystyrene standards of known molecular weights on the same column using the same solvent. A plot of the peak-elution volumes vs the product of weight-average molecular weight times the intrinsic viscosity gave an excellent fit of the methyl ester (circles) and the polystyrene standards (solid line), as shown in Fig. 6.[75] Therefore, it is now possible to relate the molecular weights of DIVEMA samples to those of polystyrene samples of known molecular weights, although admittedly the procedure is quite laborious.

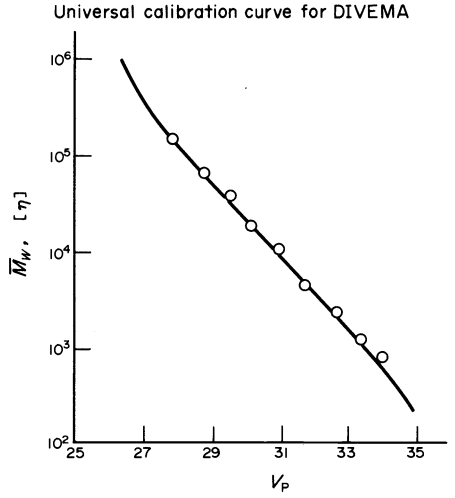


Fig. 6. Universal calibration curve for GPC using tetrahydrofuran at 30°C. ——— polystyrene standards, ○ DIVEMA methyl ester fractions.

If our hypothesis concerning activity and toxicity were correct, we would need samples of DIVEMA with narrow molecular weight distribution for evaluation, and a number of procedures were investigated for this purpose. The first used was sand-column fractionation by a modification of a procedure developed in our laboratories for fractionating polypropylene.[76] Although the procedure seemed to work quite well, it turned out to be highly irreproducible, probably because of differing degrees of hydrolysis, both of the starting material and during the fractionation. Fractional precipitation of the anhydride or the acid,[77]† as well as ultrafiltration and degradation, showed some promise for obtaining narrow molecular weight fractions, but they all suffered from yielding too little material for evaluation. We therefore turned to solution polymerization, since, if the polymer remains in solution and the polymerization is not carried to too high a conversion, an $\bar{M}_w/\bar{M}_n$ of 2 should be obtainable. The first procedure which appeared promising was photopolymerization at low temperature in tetrahydrofuran, which is a solvent for the polymer only below room temperature. No sensitizer is required for the photochemical initiation, probably because maleic anhydride forms a charge-transfer complex with the solvent, which absorbs in the ultraviolet. However, tetrahydrofuran is an active chain-transfer agent and it was difficult under these circumstances to obtain sufficiently high molecular weights. We therefore turned to the use of acetone, which is a much better solvent for the polymer, using tetrahydrofuran as a chain-transfer agent to control molecular weight; typical results, using 1% azobis(isobutyronitrile) as initiator at 55°, are shown in Table 12.

Table 12. Preparation of DIVEMA with narrow molecular weight distribution in acetone-tetrahydrofuran†

THF (%)	Time (hr)	Conversion (%)	$[\eta]$ (0.05 M NaCl)	$\bar{M}_w/\bar{M}_n$
5	5	37	1.6	
7.5	4	41	0.85	1.79
10	3	37	0.62	
20	3	49	0.33	

†Monomer conc. 0.9 M; initiator, 1% AIBN; temp, 55°.

The use of tetrahydrofuran as a chain-transfer agent to control molecular weight appeared to give much more reproducible results than the reported procedure[74] of varying the initiator concentration.

Table 13 shows the effect of molecular weight and molecular weight distribution on the toxicity and antitumor activity of various samples of DIVEMA. Samples A–E are narrow distribution copolymers prepared by several methods, including sand-column fractionation and solution polymerization; they are listed in order of increasing peak height molecular weight, as determined by gel permeation chromatography on the methyl esters. NSC 46015 is the clinical sample of DIVEMA, while XA124-177 is a higher molecular weight copolymer, also prepared by a slurry polymerization; as predicted, slurry polymerization does indeed lead to a broader molecular weight distribution. The next column shows the LD_{50} in mice upon intravenous injection; here too the toxicity increases with increasing molecular weight. SGPT (serum glutamic pyruvate transaminase, Sigma Frankel units) is a measure of liver damage; as can be seen, it increases with increasing molecular weight and the two broad distribution samples are definitely more toxic than the others.

The phagocytic index, which is another way of expressing the RES activity, is, as we suspected, a function of molecular weight. These results are based on the first-order rate of colloidal carbon clearance 24 hr after injection of DIVEMA, so that the initial RES activity is being measured. Here a value higher than the control, 0.044, indicates stimulation of activity, while one lower than the control indicates depression. Thus, it has been demonstrated that the biphasic response of the reticuloendothelial system can be avoided; below a molecular weight of about 15,000, DIVEMA stimulates the RES without an initial inhibition.

†Allen and Turner used sodium tetraphenylboron in this separation. Butler and Wu[74] reported toxicity problems because of residual boron in one sample.

Table 13. Biological activity of various samples of DIVEMA

Sample	$\bar{M}_p$†	$\bar{M}_w/\bar{M}_n$†	Acute toxicity‡	SGPT§	Phagocytic index¶	Endotoxin sensitisation‖ (% mortality)	Drug metabolism (% inhibition)	Antitumor Ehrlich§§ (% inhibition)	Antitumor Lewis lung¶¶ (T/C)
Control				30	0.044	0			
A	2500	1.7		34	0.105	0		66	> 130
B	3000	1.7		48	0.073	0	15††		> 171
C	3900	2.3		30		0			> 133
D	5200	1.9		54		20			125
E	6900	1.9	127	46	0.055	0	0‡‡	43	
F	14,700	1.6	131	52	0.075	0	0††	61	
G	19,600	2.2	100	93	0.014	100	30‡‡	45	
H	25,000	2.6		132		100			
I	44,800	2.3	87	96	0.010	100	57‡‡		
NSC 46015	22,500	3.7	96	> 200	0.021	100	67††	67	> 117
XA 124–177	32,200	7.2	72	> 200	0.015	100	65††	53	

All tests except Lewis lung were done on white male Swiss NYLAR mice, polymer dose 25 mg kg^{-1}, given intravenously.
†From GPC of methyl ester.
‡LD_{50} intravenously.
§Serum glutamic pyruvate transaminase, Sigma Frankel Units.
¶From first order rate of carbon clearance, 24 hr after injection.
‖Endotoxin from *Salmonella typhosa*, 3 mg kg^{-1} intravenous challenge.
††Aminopyrine.
‡‡Antipyrine.
§§Decrease in weight of tumor compared with control.
¶¶Data obtained from Drug Research and Development, Chemotherapy, NCI; mean survival time, % of control.

Table 14. Effect of DIVEMA molecular weight on toxicity and activity

Sample	$[\eta]$ (0.05 M NaCl)	$\bar{M}_p \times 10^{-3}$	Endotoxin sensitization	Macrophage activation	Antitumor activity against Lewis Lung
X19543–27	0.066		–	–	–
X18571–31	0.33	5.5	–	+	+
X18720–71	0.70	25.0	+	+	+
XA124–177	1.65	33.2	+	+	+

The next column lists the sensitization to a 3 mg/kg intravenous injection of *S. typhosa* endotoxin, and the increase in toxicity with increased molecular weight is quite apparent. Since the drugs are detoxified mainly in the liver, the effect of DIVEMA on the rate of aminopyrine or antipyrine metabolism is also a measure of liver function, and here too the toxicity increases with increasing DIVEMA molecular weight.

The last two columns show the antitumor activity of these same samples. The activity against these two tumors seems to be surprisingly independent of the molecular weight or the molecular weight distribution. (The value given for NSC 46015 against Lewis lung carcinoma is not meant to indicate that it was less active than the other samples; there is frequently a broad spread in these test results.)

Recently Kaplan, of the Medical College of Virginia, was able to show that a sample with the right viscosity and a narrow molecular weight distribution would not sensitize to endotoxin and would both activate macrophages and be active against Lewis lung carcinoma (Table 14). Lower molecular weight samples were inactive but nontoxic, whereas higher molecular weight samples were both active and toxic.[78]

Some very exciting results are being obtained by Chirigos and Mohr at the National Cancer Institute.[79] In studying the dose response of DIVEMA used as an adjuvant to cancer chemotherapy,[66] described previously (Table 10), they have found that DIVEMA is active at extremely low levels when administered to leukemic mice put into remission with BCNU. Thus, whereas the initial study used a DIVEMA dose level of 20 mg/kg in mice, they have now found activity at levels as low as 0.1 mg. Even though toxicity would be of much less concern at the 0.1–1 mg/kg level in humans than at the dose of 10 mg/kg used in the original evaluation of DIVEMA as a chemotherapeutic agent, Chirigos and Mohr have found that the lower molecular weight, less toxic copolymers also have good activity (Table 15).

Table 15. Effect of DIVEMA on LSTRA mice after BCNU treatment†

Designation	$[\eta]$ (0.05 N NaCl)	Survivors, %‡ 0.1 mg/kg§	Survivors, %‡ 5 mg/kg¶
X18720–91	0.17	40	70
X18571–92	0.30	60	70
X18720–71	0.70	70	70
X18720–39B	1.08	70	80
X18802–32	1.58	90	60
NSC 46015	0.76	70	100

†Tumor on day 0, BCNU on day 7.
‡After 81 days (controls, 0%).
§One injection on day 13.
¶Three injections on days 13, 15, 17.

CONCLUDING REMARKS

It should be quite apparent, from what has been said thus far, that there has been a tremendous amount of activity in recent years in the field of biologically active synthetic polyelectrolytes, too much, in fact, to be summarized in a brief lecture. I have chosen to discuss one polymer, the 1:2 divinyl ether-maleic anhydride copolymer (DIVEMA) in detail, rather than to give a survey of the area, both because of my close connection with it and because in its chemistry and biological activity it typifies this class of materials. I believe I can say, with considerable confidence, that in the not too far distant future it, or some related polymer, will find a place in medicine.

Acknowledgements—I am greatly indebted to Drs. A. E. Munson and W. Regelson, Medical College of Virginia, for much of the biological data described here; to E. I. Edwards and N. R. Newburg for the polymer syntheses; to S. J. Keahey, Dr. R. J. Samuels, and Dr. L. G. Bunville for the fractionations and physical data; and to Drs. M. A. Chirigos and S. J. Mohr, National Cancer Institute, NIH, and A. M. Kaplan, Medical College of Virginia, for permission to discuss their results prior to publication.

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