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Anionic Polymers and Biological Activities. Effects of Some New Polycarboxylic Acids on the Ascitic Sarcoma 180 of Mice

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Eleven new polymeric carboxylic acids with widely different solubilities in water have been synthesized. The activities of these polymers against the ascitic sarcoma 180 tumor of mice and their acute toxicities in mice have been compared with molecular parameters of the polymers such as molecular weights, charge densities, and abilities to complex calcium and magnesium ions. The maximum effectiveness of these polymers against ascitic sarcoma 180 of mice is greatest for those polymers having fewer carboxyl groups ionized at pH 7. Toxicities are lower for polymers having more carboxyl groups ionized at pH 7.

Synthetic polyelectrolytes often have drastic effects on biological systems.[1] Some polyacids are known to have antitumor[2] and antiviral[3] properties and the ability to induce the production of interferon in man and animals and the capacity to increase the immune response.[4] We hoped to increase these biological effects with new polymeric anions having structures that would optimize the chemical and physical properties of the polymers. Previous work has shown[2] that un-ionized water-soluble polymers such as poly(vinyl alcohol) have little effect on the growth rate of experimental tumors of animals, whereas polymers capable of ionizing at biological pH such as poly(acrylic acid) have significant antitumor activities. In this study we prepared polymers with varying numbers of carboxyl groups along the polymer chain in order to quantitate any relationship between the density of charges in the polymers and their antitumor activities. To accomplish this we prepared copolymers of (1) acrylic acid with isobutyl vinyl ether and (2) acrylic acid and itaconic acid or β-(*N*,*N*-dimethylamino)ethyl methacrylate. Since a possible mechanism of antitumor action of these polymers involves their interaction with calcium and magnesium ions, we also proposed to measure their abilities to complex calcium and magnesium ions and to correlate these properties with their antitumor activities.

Results and Discussion

Synthesis and Characterization of Polymers. Eleven new polymers containing carboxyl groups in their side chains have been synthesized and purified by methods given in the Experimental Section and in Table I. The five copolymers of acrylic acid and isobutyl vinyl ether presented special problems of synthesis. Acrylic acid is easily polymerized with free-radical initiators such as benzoyl peroxide. Pure isobutyl vinyl ether produces only oligomers with radical initiation[5] and requires the use of a cationic initiator such as aluminum acid sulfate to produce macromolecules. Therefore, a much greater portion of isobutyl vinyl ether was required in the reaction mixture in order to obtain the desired ratio of monomers in the copolymer; details are given in Table I.

Chemical compositions of the polymers, confirmed by chemical analysis and by NMR and infrared spectrometry, are shown in Table II. The NMR spectrum of poly(acrylic acid) (I) in D_2O shows three absorption peaks, a sharp singlet at δ 5.20 for the H–OD, a broad peak ca. δ 2.83 for the methine protons on the polymer chain, and a broad peak ca. δ 2.25 for the methylene proton on the polymer chain, all in agreement with the results of Sewell.[6] Integration of these peaks gave values of 1:1:2. The NMR spectrum of poly(isobutyl vinyl ether) (VII) in carbon tetrachloride shows a doublet at δ 0.91 with a coupling constant of 6 Hz. The absorption of other protons of VII all merge into two broad peaks around δ 3.41–3.09 and 1.69.

Polymer VIII, poly(itaconic acid), has an elemental analysis of C 40.66 and H 5.01, corresponding roughly to a formula of $C_5H_6O_4 \cdot 0.7H_2O$ for which the calculated values are C 42.08 and H 5.19. The NMR spectrum of VIII in D_2O shows the ratio of the H–OD to all other protons as 3.3:4.0; the corresponding ratio for the above formula is

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Table I. Synthesis and Purification of Copolymers of Acrylic Acid and Isobutyl Vinyl Ether

no.	polymer	reaction conditions: molar ratio of monomers	temp, °C	time, h	purification	yield, %
II	poly(acrylic acid–isobutyl vinyl ether)	0.25[a]	82	1	solid washed with ligroine	72
III	poly(acrylic acid–isobutyl vinyl ether)	0.51[a]	70	8	solid washed with benzene	52
IV	poly(acrylic acid–isobutyl vinyl ether)	0.67[a]	82	1	acetone–benzene soln added to ligroine	69
V	poly(acrylic acid–isobutyl vinyl ether)	1.5[a]	82	1	acetone–benzene soln added to 0.05 N HCl	60
VI	poly(acrylic acid–isobutyl vinyl ether)	3.8[a]	82	1	acetone–benzene soln added to methanol–water	42

[a] Isobutyl vinyl ether–acrylic acid.

Table II. New Polymers

polymer	formula[a]	mol wt × 10^{-4} [c]	pK_{av}	carboxyl groups ionized at pH 7, %	charge density, at pH 7, charges/2 C in chain
I	(AA)	25	6.58	62	0.62
II	$(AA)_x \cdot (IBVE)_{0.035x}$	25	6.69	58	0.56
III	$(AA)_x \cdot (IBVE)_{0.046x}$	8.5	6.70	56	0.54
IV	$(AA)_x \cdot (IBVE)_{0.29x}$	25	6.67	58	0.45
V	$(AA)_x \cdot (IBVE)_{0.43x}$	25	6.28[d]	73	0.51
VI	$(AA)_x \cdot (IBVE)_{0.73x}$	12	6.27[d]	77	0.44
VII	$(IBVE)_x$	[b]	[b]	[b]	[b]
VIII	$(ITCA)_x \cdot (H_2O)_{0.7x}$	8.7	8.7	35	0.70
IX	$(AA)_x \cdot (ITCA)_x \cdot (H_2O)_{0.4x}$	4.4	7.7	37	0.54
X	$(AA)_x \cdot (DMAEM)_x$	[b]	[b]	77	0.38
XI	$(AA)_x \cdot (IOVE)_{0.15x}$	4.9	6.63	59	0.51

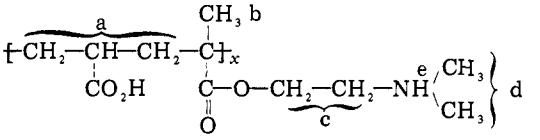
[a] AA = acrylic acid; IBVE = isobutyl vinyl ether; ITCA = itaconic acid; DMAEM = β-(*N,N*-dimethylamino)ethyl methacrylate; IOVE = isooctyl vinyl ether. From (1) titration with base, (2) chemical analysis, and (3) integration of NMR spectra. [b] Insoluble in water. [c] From viscosity measurements. [d] Salt added to the solution.

3.4:4.0. The difficulty of removing water from poly(itaconic acid) has been reported.[7] The NMR spectra of the poly(methyl acrylates) (XII and XIII) prepared by two different methods are similar except for a sharp singlet at δ 2.12 which appears in the spectrum of XII. This singlet is attributed to the greater tacticity achieved by polymerizing methyl acrylate as compared to acrylic acid. The infrared spectrum of poly(isobutyl vinyl ether) (VII) agrees with that of Natta[8] except for a sharp, weak absorption at 1710 cm^{-1} that corresponds to a C=C stretching vibration that may be caused by thermal depolymerization of the film as it dried at 110 °C.

The ratio of monomers in the copolymers was determined by (1) potentiometric titration with standard NaOH, (2) chemical analyses for carbon and hydrogen (and nitrogen when applicable), and (3) integration of NMR peaks for certain hydrogen atoms. Since polymers containing carboxyl groups do not give distinct breaks at the end points in plots of pH vs. volume of base added,[9] the calculation of equivalent weights of the polymers by this method was not so precise as desired. However, the ratio of monomers in the polymers could be corroborated by elemental analysis and NMR.

Potentiometric titration of the copolymers of acrylic acid and isobutyl vinyl ether (II–VI) gave equivalent weights corresponding to the ratio of the two monomer units in the polymer. The copolymers having high proportions of isobutyl vinyl ether dissolved with difficulty in water but dissolved readily in dilute base. The copolymer of acrylic acid and itaconic acid, IX, from its elemental analysis of C 46.62 and H 5.62 can be represented by the formula $C_3H_4O_2 \cdot C_5H_6O_4 \cdot 0.4H_2O$ for which the calculated values are C 45.89 and H 5.16. The NMR spectrum of IX in D_2O

Table III. NMR Spectrum of Polymer X



proton	δ	integration	description
H_a	1.8–2.6	5 H	broad
H_b	1.26	3 H	broad
H_c	~3.8	4 H	broad
H_d	3.22	6 H	doublet, J = 9 Hz
H_e	4.52	~1 H	broad

gives a ratio of H–OD to polymer protons of 3.9 to 7 compared to 3.8 to 7 for the proposed formula. The NMR spectrum in formic acid of X, summarized in Table III, indicates that this copolymer of acrylic acid and β-(*N,N*-dimethylamino)ethyl methacrylate has a monomer ratio of 1:1. The equivalent weight of XI, calculated on the basis of the potentiometric titration with base, is 95.1, indicating a ratio of monomer units of 0.15 for isooctyl vinyl ether–acrylic acid.

Molecular weight averages of the compounds were determined by viscosity measurements. Since the viscosity of polymeric acids is affected by the pH of the solution,[10] all determinations were made with buffered solutions, usually at pH 7. Number-average molecular weights of polymers XII and XIII were determined in acetone solution by vapor pressure lowering as 2.33×10^4 and 2.15×10^4. Since the weight-average molecular weight of XIII by viscosity measurements is 3.6×10^5, the $\bar{M}_n/\bar{M}_w$ ratio has a value of 0.06, indicating a wide spread in molecular

Table IV. Precipitation of Polymers by Calcium and Magnesium Ions in Water at pH 7 and 25 °C

	calcium ions			magnesium ions			
polymer	unbound, mol	β	β'	unbound, mol	β	β'	unbound metal ions, Mg^{2+}/Ca^{2+}
I	0.0	0.18	0.29	0.25	0.18	0.29	∞
II	0.0	0.18	0.31	0.18	0.18	0.31	∞
III	0.09	0.16	0.29	0.28	0.20	0.36	3.1
IV	0.88	0.38	0.66	1.66	0.25	0.43	1.89
V	did not precipitate			did not precipitate			
VI	did not precipitate			did not precipitate			
VII	not soluble at pH 7			not soluble at pH 7			
VIII	0.35	0.25	0.71	did not precipitate			
IX	0.23	0.13	0.35	0.55	0.28	0.76	2.39
X	0.11	0.054	0.070	0.17	0.093	0.12	1.55
XI	0.06	0.15	0.25	0.29	0.32	0.54	4.83

weights for polymers I and XIII.[11]

The reduced viscosities of these polyanions at 25 °C and pH 7 were found to rise sharply as their concentrations were reduced, in accordance with the Fuoss equation[12] which was developed for other polyelectrolytes

$$Z = \frac{A}{1 + Bc^{1/2}} + D \quad (1)$$

where Z is the reduced viscosity (specific viscosity/concentration), c is the concentration of polymer, A is a constant which depends on the molecular weight of the polymer and the extent to which the polymer coil can expand at infinite dilution owing to the intramolecular coulomb repulsion between charges attached to the chain, B is a constant that increases with decreasing dielectric constant of the solvent, and D is the parameter for linearizing the plot of $1/(Z - D)$ vs. $c^{1/2}$. For all polymers studied in this work, the value of D was found to be 0 since plots of the reciprocal of the intrinsic viscosity vs. $c^{1/2}$ gave straight lines with slopes of B/A and intercepts of $1/A$.

The value of A at infinite dilution is known to be the intrinsic viscosity, which is related to the molecular weights of the polyelectrolytes. Liberti and Stivala[13] found that A for heparin is proportional to $M^{1.59}$, where M is the molecular weight. Strauss and Smith[14] found that A is proportional to $M^{1.87}$ for polyphosphates. For an approximate comparison of molecular weights for these polymeric electrolytes, an A value proportional to $M^{1.7}$ was assumed. From the data measured for polymer I at pH 7 and 25 °C, the proportionality constant between A and $M^{1.7}$ is 1.3×10^{-7}. This constant was used to calculate the molecular weights of the other polyelectrolytes that are listed in Table II.

Chemical Studies. Potentiometric titrations of the polymers gave the extent of dissociation of the carboxyl groups at pH 7.0, the average pK_a, and the charge density along the chain (Table II). As the carboxyl groups became more separated by the isobutyl vinyl ether groups, the extent of dissociation at pH 7.0 increases. The charge density along the chain decreases as more isobutyl vinyl ether groups are introduced into the copolymer but the density of charges is partially offset by the increased extent of dissociation caused by greater separation of the carboxyl groups.

Turbidimetric methods were used to measure the complexation of calcium and magnesium ions by these polymers (Table IV). When divalent cations are added to solutions of polyanions, the concentrations at the point of precipitation are given by the equation[15]

$$[M^{2+}]_{total} = [M^{2+}]_{free} + \beta[-CO_2H]_{total} \quad (2)$$

where $[M^{2+}]_{total}$ is the total divalent metal ion concentration, $[M^{2+}]_{free}$ is the free divalent metal ion concentration, β is the number of equivalents of divalent metal ions bound per acid group, and $[-CO_2H]_{total}$ is the total concentration of acid groups. When $[M^{2+}]_{total}$ is plotted vs. $[-CO_2H]_{total}$ the concentration of $[M^{2+}]_{free}$ appears as the intercept and β as the slope of the plot. Since in most cases these polyacids were not completely ionized, the equivalents of the metal ion bound to each ionized carboxylate group, β', were calculated[15] by eq 3 where β is the

$$\beta' = \beta/\alpha \quad (3)$$

slope obtained from the previous plot and α is the degree of ionization. There results are tabulated in Table IV for the polymers studied.

These polymers differ in their abilities to complex magnesium and calcium ions as shown by the values of β' in Table II. Copolymers of acrylic acid and isobutyl vinyl ether (II–IV) complex calcium and magnesium ions better as the proportion of isobutyl vinyl ether increases. However, the ability of these copolymers to complex calcium ions increases more rapidly than their ability to complex magnesium ions as the proportion of isobutyl vinyl ether in the copolymer becomes greater. The concentration of unbound magnesium ions at the point of precipitation is greater than the concentration of calcium ions, but this ratio decreases as the proportion of isobutyl vinyl ether in the copolymer increases. The two copolymers of acrylic acid and isobutyl vinyl ether with the highest proportion of isobutyl vinyl ether complexed calcium and magnesium so well that no precipitate was formed under the conditions used. The homopolymer of itaconic acid, VIII, complexed calcium ions very well and magnesium ions even better. The copolymer of acrylic acid and itaconic acid, IV, also complexed magnesium ions better than calcium ions.

Biological Studies. The acute toxicities of these compounds in Swiss mice were determined by injection of single doses (Table V). Polymers VIII–X are least toxic to Swiss mice under these conditions. These polymers also have more than two carboxyl groups for each four carbon atoms of the chain. Polymer III, with a molecular weight of 85 000, has less toxicity than similar polymers II and IV, with molecular weights of 250 000. Low toxicity appears to be associated with polymers of low molecular weight and/or many carboxyl groups.

The abilities of these polymers to slow the growth of the ascitic sarcoma 180 tumor of mice were determined by the protocol described in the Experimental Section with the results shown in Table V. The ratio (T/C) of the survival times (T) of the treated animals and the survival times of the control animals (C) is used as a measure of the effectiveness of the treatment. An increase in average survival times for six animals of 25% (T/C, 125%) is considered to be statistically significant of antitumor

Table V. Toxicities and Antitumor Effects

polymer	toxicity in male Swiss mice,[a] LD_{50}, mg/kg	effect on ascitic sarcoma 180 in female Swiss mice: dose,[b] mg/kg	survivors at 7 days	av wt change: treated, %	av wt change: controls, %	survival times, av ± SE: treated, days	survival times, av ± SE: controls, days	T/C,[c] %
I	39	12	6/6	+8.8	+12.2	14.8 ± 3.0	11.2 ± 2.6	132
II	25	10	6/6	+8.1	+7.6	15.3 ± 1.6	9.8 ± 3.2	156
III	82	12	6/6	+4.3	+20.1	15.7 ± 5.2	9.2 ± 2.6	171
IV	13	4.5	6/6	−2.5	+13.1	17.5 ± 5.2	11.7 ± 3.5	150
V	3.5	1.9	6/6	+3.5	+35.0	13.2 ± 2.1	11.8 ± 4.1	111
VI	3.5	1.2	6/6	+8.7	+7.6	12.0 ± 1.3	9.8 ± 3.2	122
VIII	425	200	6/6	−0.9	+12.2	16.2 ± 2.3	11.2 ± 2.6	145
IX	300	100	6/6	−6.1	+7.6	18.8 ± 3.7	9.8 ± 3.2	192
X	600	175	6/6	+3.7	+12.2	16.2 ± 4.9	11.2 ± 2.6	145
XI	10.5	7.0	6/6	−8.7	+7.6	12.3 ± 2.2	9.8 ± 3.2	125

[a] The single ip dose that will kill approximately 50% of the animals within 7 days. [b] Three doses administered ip on alternate days, starting 24 h after ip injection of ascitic sarcoma 180 cells. [c] Ratio of the average survival time of the treated mice (T) to that of the control mice (C).

activity for several tumor systems according to criteria used by the National Cancer Institute.[16] Eight of these polymers caused an increase in average survival times of at least 25% as compared to the controls. Polymer IX with a T/C value of 192%, is the most active of these polymers in this tumor system. In general, polymers with many carboxyl groups attached to the carbon chain have significant antitumor activity in this system.

The therapeutic index, defined in this study as the ratio of the LD_{50} to the optimum dose, is an important criterion for any drug. Among these compounds polymer III has the best therapeutic index. With a T/C value of 171% at an optimum dose of 12 mg/kg and an LD_{50} in mice of 82 mg/kg, its therapeutic index is 6.8. Although polymer III is not as effective as polymer IX on the basis of the T/C value, it is more effective based on the smaller dose required.

Discriminant Analysis. The results were subjected to discriminant analysis in order to determine which molecular factors were most significant in classifying the polymers as to antitumor activity and toxicity. The most significant variable for distinguishing between the polymers according to their LD_{50} and the optimum dose is the percent of carboxyl groups ionized at pH 7. Although the confidence level of the discrimination is not high (less than 80%), it appears that an increase in this variable results in a reduction in the optimum dose level and an increase in the toxicity. The most significant variable in determining the maximum T/C values is also the percent of carboxyl groups ionized at pH 7. This variable alone can classify correctly nine of the ten polymers studied at a confidence level of 97.5%. Compounds with higher T/C values are characterized by lower percentages of carboxyl groups ionized. The therapeutic index defined as the ratio of the LD_{50} to the optimum dose is most significantly related to the magnesium ions complexed per carboxyl group for the seven compounds for which these values are known. The significance level is 90% and five of the seven compounds are classified correctly by this variable.

Conclusions

Polymers with many carboxyl groups should be desirable antitumor agents because of their low toxicities and their effectiveness against the ascitic sarcoma 180 of mice. The therapeutic index of these polymers as antitumor agents may be related to their ability to complex magnesium ions.

Experimental Section

Preparation and Purification of Poly(acrylic acid) (I). The method used for this preparation was adapted from the patent by Barrett.[77] Acrylic acid (5.00 g, 0.0694 mol), benzoyl peroxide (0.005 g, 0.02 mmol), and benzene (100 mL) were added to a 500-mL three-neck round-bottom flask equipped with a condenser and a mechanical stirrer. The mixture under dry nitrogen was heated with an oil bath at 82 °C. After polymerization started, a mixture of 36.0 g (0.50 mol) of acrylic acid, 0.30 g (1.2 mmol) of benzoyl peroxide, and 110 mL of benzene was added continuously through a dropping funnel to the reaction flask during 12 h. The poly(acrylic acid), which precipitated from the benzene solution, was collected by filtration. The product was washed in a blender three times each with 200 mL of benzene and filtered out each time. The polymer was then dried under reduced pressure at room temperature. The yield was 36.3 g, 89.0% of theory.

Copolymers of Acrylic Acid and Isobutyl Vinyl Ether (II–VI). These copolymers were prepared from acrylic acid and isobutyl vinyl ether in various ratios using benzoyl peroxide as the catalyst and benzene as the solvent. The procedure for the synthesis of II is given here. Other copolymers were prepared in a similar manner with details given in Table I.

Acrylic Acid–Isobutyl Vinyl Ether Copolymer (II). Acrylic acid (58.2 g, 0.81 mol), isobutyl vinyl ether (20.2 g, 0.20 mol), and benzoyl peroxide (0.726 g, 3 mmol) were added to 200 mL of benzene in a 1000-mL three-neck flat-bottom reaction flask equipped with a condenser and a mechanical stirrer. The polymerizing mixture was kept under dry nitrogen and heated to 82 °C with an oil bath. The initiation time was about 20 min and the reaction was completed after stirring and heating for 1 h. The mixture was filtered and the solid transferred to a blender where it was washed three times each with 200 mL of ligroine. The solid was then dried under reduced pressure at room temperature. The yield was 56.0 g, 71.4% of the theoretical yield.

Poly(isobutyl vinyl ether) (VIII). The catalyst for this polymerization was prepared by dissolving 10.0 g (0.015 mol) of $Al_2(SO_4)_3 \cdot 18H_2O$ in the minimum amount of water.[18] Concentrated sulfuric acid (3.30 g) was added and the solution was evaporated to dryness. The residue was placed in an oven at 170 °C for 4 h. A sample of the dried salt (0.20 g) was suspended in 10.0 mL of mineral oil to give a 2% suspension of $Al_2(SO_4)_3 \cdot 3H_2SO_4 \cdot 7H_2O$.

Isobutyl vinyl ether (19.49 g, 0.19 mol) and 150 mL of *n*-pentane were placed in a 16-oz pressure bottle. The air in the bottle was replaced by dry nitrogen and 0.5 mL of the catalyst suspension was added through a syringe. The mixture was heated in a water bath at 35 °C for 47 h. The polymer was precipitated in methanol in the presence of a small amout of *N*-phenyl-2-naphthylamine. The precipitate was washed in 150 mL of methanol three times in a blender. The solid collected through filtration was dissolved in benzene and then freeze-dried. The yield was 15.3 g, 79.0% of theory.

Poly(itaconic acid) (VIII).[19] Itaconic acid (50.0 g, 0.38 mol), potassium peroxydisulfate ($K_2S_2O_8$) (0.25 g, 0.9 mmol), and 125 mL of 0.5 N hydrochloric acid were added to a 12-oz beverage bottle. The air in the bottle was replaced by dry nitrogen. Polymerization was carried out at 50 °C by heating in an oil bath under stirring for 148 h. The polymer was precipitated by adding

the reaction mixture dropwise to 1400 mL of acetone with vigorous stirring. The polymer was collected by filtration. The final solid was dried under reduced pressure of 50 °C and ground to a powder. The yield was 14.5 g, 29.0% of theory.

Acrylic Acid–Itaconic Acid Copolymer (IX).[19] Acrylic acid (20.0 g, 0.28 mol), itaconic acid (20.0 g, 0.15 mol), and potassium peroxydisulfate (0.20 g, 0.7 mmol) were dissolved in 100 mL of water and transferred to a 16-oz pressure bottle. The air in the bottle was replaced by dry nitrogen and the bottle was shaken with a mechanical shaker at room temperature for 8 days. The polymer was precipitated by adding the viscous water solution slowly to acetone (volume ratio = 1:8) with stirring. The polymer was redissolved in water and precipitated again in acetone. The polymer was then dried in a rotary evaporator under reduced pressure at 50 °C and the residue was ground to a powder. The yield was 27.5 g, 68.8% of theory.

Acrylic Acid–*N*,*N*-Dimethylaminoethyl Methacrylate Copolymer (X). Acrylic acid (25.0 g, 0.35 mol) and *N*,*N*-dimethylaminoethyl methacrylate (25.0 g, 0.175 mol) were copolymerized with potassium peroxydisulfate (0.500 g, 1.85 mmol) in 400 mL of water under dry nitrogen at room temperature for 124 h. The polymer was precipitated by adding the water solution slowly to acetone (volume ratio = 1:10) with stirring. The solid was washed in a blender three times each with 200 mL of methanol. The final polymer was collected by filtration and dried under reduced pressure at room temperature. The yield was 45.8 g, 91.6% of theory.

Acrylic Acid–Isooctyl Vinyl Ether Copolymer (XI). Acrylic acid (25.0 g, 0.175 mol), isooctyl vinyl ether (25.0 g, 0.16 mmol), benzoyl peroxide (0.500 g, 2.1 mmol), and 390 mL of benzene were kept at 53 °C under dry nitrogen for 4 h. The polymer was precipitated by adding the benzene solution slowly to ligroine (volume ratio = 1:3) with stirring. The polymer was washed three times in a blender each time with 200 mL of ligroine, filtered out, and dried under reduced pressure at room temperature. The dry polymer was a fine white powder. The yield was 32.3 g, 64.6% of theory.

Poly(methyl acrylate) (XII). Methyl acrylate (19.1 g, 0.22 mol), benzene (50.0 mL), and 2,2-azobis(isobutyronitrile) (0.058 g, 0.35 mmol) were added to a screw-capped vial and the air in the vinyl was replaced by dry nitrogen. The mixture was stirred and heated at 60 °C for 20 h. The polymer was precipitated in methanol (1 vol of benzene solution to 10 vol of methanol) with stirring. The polymer was redissolved in tetrahydrofuran and precipitated again in methanol (1:10 volume ratio). The polymer was dissolved in 50 mL of benzene and freeze-dried under reduced pressure. The yield was 15.5 g, 81.2% of theory.

Esterification of Poly(acrylic acid) to Give Poly(methyl acrylate) (XIII). Sodium hydroxide (2.4 g, 0.06 mol) was dissolved in 20 mL of water. This solution was mixed with 50 mL of 2-(2-ethoxyethoxy)ethanol and 150 mL of anhydrous ethyl ether. *N*,*N*′-Dinitroso-*N*,*N*′-dimethylterephthalamide (7.1 g, 0.036 mol) was added to the mixture. The diazomethane thus generated was distilled with ether by heating over a steam bath to a collector cooled with dry ice.[20] The ether solution of diazomethane was poured into an Erlenmeyer flask containing 3.0 g (containing 0.042 mol of CO_2H) of poly(acrylic acid) (I). Diazomethane solution was added until the yellow color persisted in the flask. The flask was warmed over a hot plate to decompose the unreacted diazomethane. All ether was then evaporated. Chloroform (30 mL) was added to dissolve the polymer. The polymer was precipitated in 450 mL of hexane. The polymer was redissolved in 50 mL of ethyl acetate and was precipitated again in 1200 mL of methanol. The residue was dissolved in 25 mL of benzene and was freeze-dried under reduced pressure. The yield was 2.94 g, 82% of theory.

Characterization of Polymers. Chemical analyses were made by M-H-W Laboratories, Garden City, Mich. For NMR studies the polymers were dissolved in dimethyl-d_6 sulfoxide, acetone-d_6, water-d_2, formic acid, CCl_4, or chloroform-*d*. The solutions were examined on a Varian Associates Model A-60 or Model XL-100 nuclear magnetic resonance spectrometer. For infrared absorption studies each polymer was dissolved in an appropriate solvent, the solution was placed on a salt plate, and the solvent was evaporated. The spectrum was obtained on a Beckman 5 infrared absorption spectrometer.

Potentiometric titrations were performed with a Beckman Research pH meter equipped with a Brinkman Instrument heater-circulator and thermoelectric cooler for constant temperature control. Since the plots of pH vs. volume of base added did not show sharp changes at the end point, pH 7.0 was used as the end point. These plots could be converted to straight lines by plotting pH vs. log $[(1 - \alpha)/\alpha]$, where α is the percent of ionization (represented by the ratio of base added to the total base required). These values are listed in Table II as the percent of carboxyl groups ionized at pH 7.0. The expanded Henderson–Hasselbalch equation was used to calculate the pK_{av} for each polymer

$$\mathrm{pH} = pK_{av} - n \log [(1 - \alpha)/\alpha] \quad (4)$$

where pK_{av} is the value of the average dissociation constant for each polymer, n is an empirical parameter, and α is again the degree of dissociation.[21] Values of pK_{av} are listed in Table II. The charge density along the polymer chain is the product of the percent of carboxyl groups ionized and the percent of acrylic acid in the polymer molecule (Table II). Molecular weight averages of the compounds were determined by viscosity measurements in water solution using Cannon–Fenske viscometers and a constant temperature of 25 °C. The polymers were dissolved (when possible) in water and brought to pH 7.0 with NaOH. At least three concentrations of each polymer were used in order to determine the intrinsic viscosity,[22] $[\eta]$, by eq 5 and 6; η_{sp} is the specific viscosity, and c is the concentration of polymer in g/100 mL. Molecular weight averages of polymers XII and XIII were

$$\eta_{sp}/c = (\eta_{sp}/c)_0(1 + B_1 c) \quad (5)$$

$$[\eta] = (\eta_{sp}/c)_0 \quad (6)$$

determined on their acetone solutions with a Coleman 115 molecular apparatus. Results of these studies are shown in Table II.

Turbidity studies were performed with a Cary Model 14 spectrophotometer and a "titration head", a specially designed motor-driven buret of variable and reproducible rate of titrant delivery and a special cell holder comprising an air-driven stirrer.[23] Aqueous polymer solutions, 0.01–0.10 M in CO_2H groups, were added to the stirred cell at 25 °C and the latter was placed in the spectrometer. Solutions containing calcium and magnesium ions of known concentrations were added by means of the "titration head". The formation of turbidity in the polymer solution was indicated by a sharp change in the optical density at 505.0 nm. The concentration of calcium and magnesium ions at the point of precipitation was calculated from the volume of these solutions added to the cell. When the concentration of metal ions at the point of precipitation was plotted against various concentrations of carboxyl groups for a particular polymer, straight lines were obtained for each polymer and each metal. The intercept represents the concentration of free metal ion and the slope, β, represents the equivalents of metal ions bound to each CO_2H group. The equivalents of metal ion bound to each carboxylate ion, β', was calculated by eq 3. Results are shown in Table IV.

Bioassay. The LD_{50} for a single dose of each polymer was determined in male Swiss mice by single ip injections of aqueous solutions of the polymers that had been neutralized to the end point of phenolphthalein with sodium hydroxide. Six mice were used for each test and survivors were counted and weighed 5 days later. The results are shown in Table V.

Ascitic sarcoma 180 studies were made with female Swiss mice supplied by ARS/Sprague–Dawley, Madison, Wis., using ascitic sarcoma 180 cells and a protocol originally furnished by Frederic A. French of Mt. Zion Hospital and Medical Center, San Francisco, Calif.[24] Mice were each given an ip injection of a neutral aqueous solution of the compound under test on days 1, 3, and 5. At least six mice were used for each control and test set; the survival times of the animals in each set were averaged. The average weight gains of live animals on day 7 were determined for treated and control animals.

Discriminant Analysis. The polymers were divided into two classes as equally as possible based on the LD_{50}, the optimum dose, the T/C values, and the therapeutic index. Discriminant analysis was performed using as discriminatory variables the molecular parameters shown in Tables II and IV. The BMD07M stepwise

discriminant analysis program of the Biomedical Computer Programs was used to find the most significant variable that best distinguishes between the two groups based on these four factors. This program, developed under grant FR-3 of the Division of Research Facilities of the National Institutes of Health, can be used without great difficulty on a modern computer. Documentation describing the mathematical steps and the procedure for using the program are available.[25]

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