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Synthesis and Properties of Novel Polyanions of Potential Antitumor Activity

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Synthesis and Properties of Novel Polyanions of Potential Antitumor Activity

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ABSTRACT

A large number of naturally occurring polyanionic materials are known each of which possesses its own set of physiological properties. Within recent years, a wide variety of synthetic polyanions have become available, and investigators have compared their physiological properties with the naturally occurring polyanions. Perhaps, the most widely investigated synthetic polyanion is the 1:2 regularly alternating cyclocopolymer (DVE-MA) of divinyl ether (DVE) and maleic anhydride (MA), first discovered in these laboratories in 1951. This material has been investigated extensively, both from the standpoint of its chemical structure and its biological activity. Although certain aspects of its structure remain to be determined, it has been shown to possess a wide spectrum of biological activity. It possesses antitumor as well as other activities and is an interferon inducer. Many structural modifications of DVE-MA have been synthesized which also possess antitumor activity and interferon-inducing capability. A recent structural modification incorporated 5-fluorouracil (5-FU) into DVE-MA. This polymer is hydrolyzed readily to release 5-FU, and the material exhibited considerable activity against P388 lymphocytic leukemia. A novel series of polyanions have been synthesized via reactions

of N-substituted triazolinediones with a variety of olefins and diene polymers. The synthesis and physiological properties of certain of these polyanions are discussed in this paper.

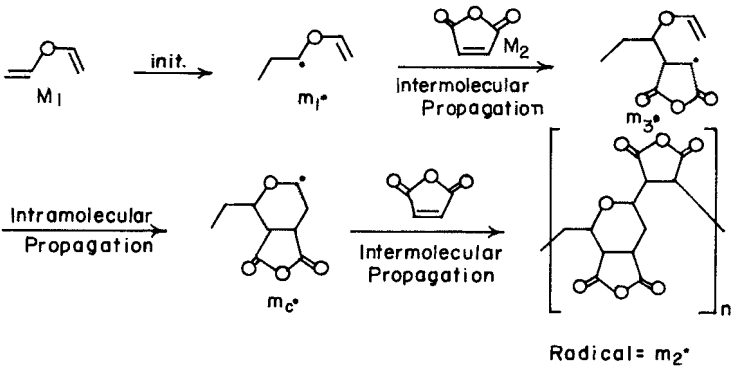
INTRODUCTION

Synthetic biologically active polymers have elicited considerable interest in recent years. Although there are a large number of naturally occurring polymers possessing polyelectrolyte character, polycationic materials are relatively few. On the other hand a large number of naturally occurring polyanions are known. Among these are such proteins as chondroitin sulfate, DNA, heparin, hyaluronic acid, and RNA, and such plant gums as agar, alginic acid, carrageenin, gum arabic, gum tragacanth, and pectin. Within recent years, a wide variety of synthetic polyanions have become available and investigators have compared their physiological properties with those of naturally occurring polyanions. One of the first polyanions to be investigated extensively was sodium ethylenesulfonate [1, 2] which was shown to possess antitumor activity in mice against adenocarcinoma 775, Ehrlich (ascites), Krebs 2 carcinoma (ascites), L1210 lymphoid leukemia, L5178 lymphoid leukemia, and Sarcoma 180. These observations were followed by investigations which showed that a number of polycarboxylic acids, such as poly(acrylic acid), poly(methacrylic acid) and the ethylene-maleic anhydride copolymer possessed similar activity to that of sodium ethylene-sulfonate [3]; however, the materials exhibited undesirable toxicity.

PHYSIOLOGICAL PROPERTIES AND STRUCTURAL STUDIES OF THE COPOLYMER OF DIVINYL ETHER AND MALEIC ANHYDRIDE

Physiological Properties

Perhaps one of the most widely investigated polyanions has been the hydrolyzed form of the 1:2 regularly alternating cyclocopolymer (DVE-MA) of divinyl ether (DVE) and maleic anhydride (MA), commonly known as "Pyran Copolymer" [4]. This copolymer has been of special interest to us, since it was first synthesized in 1951, characterized and subjected to various chemical studies in these laboratories [5-9]. The results of Regelson et al. [2, 3] prompted us to submit DVE-MA of $\overline{M}_n$ = 15,000-20,000 to the Cancer Chemotherapy National Service Center, National Institutes of Health, for



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evaluation as a potential antitumor agent. The results of this study [10] showed that in one test, the tumor weight developed by the test animals was only 11% of that of the control animals.

In an unusual example of duplicative effort, it became apparent in 1965 that "quite an active material–supplied from an industrial company" and designated NSC-46015, "pyran-2-succinic anhydride, 4,5-carboxytetrahydro-6-methylene dianhydride" was the same material as DVE-MA [11], the structure of which had been published from these laboratories [5-9]. Further inquiry [12] revealed that the above sample had been submitted as a " 'commercial discreat material' and was so classified until recently when Hercules Powder Company allowed us to declassify their material." Although the structure of DVE-MA had been supplied to NIH [10], it was further stated "we did not have sufficient information on the polymer supplied . . . to assign a rigorous structure, thus it was carried in our files with no correlation to the Hercules polymer" [12].

The results of one test with NSC-46015 comparable to that reported above for DVE-MA showed that the tumor weight developed by the test animals was 14% of that of the control animals. Based upon these and additional tests, DVE-MA, designated NSC-46015, was approved for clinical evaluation and was found to possess the ability to induce the generation of interferon [13, 14].

Interferon, first discovered in 1957 [15], is a substance of protein-like structure which is produced in the cells of vertebrates in response to viral infection, and possesses antiviral action. Today, it is generally accepted that interferon has an essential role in the formation of a host's nonspecific resistance to superinfection with a second virus [16]. Thus, the role of interferon in combating viral infections may be similar to that of antibodies toward bacterial infections. It is immediately apparent that a successful and readily available interferon inducer, such as the divinyl ether-maleic anhydride cyclocopolymer

(DVE-MA) may be, could play an extremely significant role not only in aiding in recovery from a viral infection, but in its use in prior generation of interferon to prevent a viral attack.

The results of the extensive biological evaluations of Pyran Copolymer have been summarized [17].

Copolymers related in structure to DVE-MA which have shown antitumor activity [18] are the half amide of the 1:1 copolymer of itaconic anhydride and furan, and the 1:1 copolymer of maleic anhydride and furan [19].

In evaluating these copolymers, L-1210 lymphoid leukemia cells were injected into test animals by intraperitoneal route on day zero. Dosages of the test drug were calculated on a milligram per kilogram body weight basis, dissolved in saline, and injected by the intraperitoneal route on day one. The mean survival time in days of the test group and the control group was calculated, and a ratio of test animals to control animals (T/C) was calculated. In all tests, the animals were evaluated at five days, for survival, as a measure of drug toxicity. All data presented in this paper represent 6/6 survivors in each test group. The evaluation results on the former structure showed that a dosage of 600 mg/kg, T/C(%) = 148. The results on the latter structure showed that at a dosage of 400 mg/kg, T/C(%) = 127. By similar test methods [18], the 1:2 copolymer of divinyl ether and citraconic anhydride and the half amide of the 1:1 copolymer of maleic anhydride and furan [20] gave the following results, respectively: at a dosage of 400 mg/kg, T/C(%) = 130; at 400 mg/kg, T/C(%) = 126.

The antitumor activity of DVE-MA is demonstrated by its effectiveness against adenocarcinoma 755, Lewis lung carcinoma, Friend leukemia virus, and Dunning ascites leukemia [17]. The respective minimum effective doses for the above were 10, 4, 10, and 7.5 mg/kg, and the respective therapeutic indexes (maximum tolerated dose/minimum effective dose) were 4, 8, 8, and 8. A comparison of the activity of DVE-MA against Lewis lung carcinoma, a slow-growing solid tumor which is difficult to control with certain other antitumor agents [17] showed it to be comparable to cyclophosphamide, a widely used alkylating agent, and far superior to 6-mercaptopurine, an antimetabolite, which showed essentially no activity.

The broad spectrum of antiviral activity of DVE-MA is further demonstrated by its activity against Rauscher leukemia [21], Maloney sarcoma [22], vesicular stomatitis [23], Mengo [24], MM [25] and foot and mouth disease [26]. The results of the test with Maloney sarcoma are as follows [22]. Animals were given five daily injections of DVE-MA (25 mg/kg) prior to intraperitoneal injection of virus. Virus dilutions of 10^{-2} and 10^{-3} were used. At both virus dilutions most of the control mice were dead within 60 days. At virus dilution of 10^{-2}, only 40% of the treated mice had

died within 140 days; at virus dilution of 10^{-3} the mice were completely protected by DVE-MA.

The antibacterial activity of DVE-MA is demonstrated against both gram-positive [27] and gram-negative [28]. It was found that mice treated with DVE-MA became resistant to Listeria monocytogenes after 4 days and retained their resistance up to two months [27].

DVE-MA also inhibits adjuvant disease [29], a disease believed to be the result of a hypersensitivity reaction to mycobacterial antigens and has similarities to rheumatoid arthritis. Rats were protected by DVE-MA if injected at days -1 and +7, with respect to adjuvant injection but not at day +14. The latter result is due to the delayed reaction.

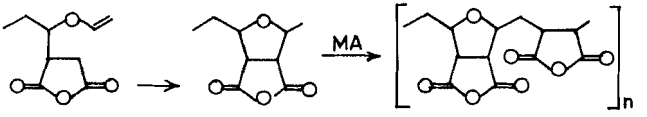
The extensive biological investigation of DVE-MA has also shown it to be antifungal [28, 30], to stimulate immune response [31-34], and to be an anticoagulant [35].

The antitumor and interferon-inducing properties of DVE-MA are molecular weight-dependent [36], and the polymer shows appreciable toxicity for molecular weights in excess of 18,000-20,000. It was shown that low molecular weight copolymers with narrow molecular weight distribution are not only low in toxicity but retain the antitumor activity shown by higher molecular weight samples against both Ehrlich adenocarcinoma and Lewis lung carcinoma in mice [37, 38].

A series of investigations is under way to study the physicochemical interactions of DVE-MA with divalent cations [39, 40]. This interaction follows an order of affinity for site binding, $Mn^{2+} > Ca^{2+} > Mg^{2+}$. The formation of a soluble chelate complex was indicated, and specific conditions in terms of pH and degree of neutralization were found under which complex formation was preceded by precipitation of microspheres. DVE-MA appears to precipitate under conditions which are close to physiologic. A mechanism was suggested for microsphere formation that is consistent with a hypothesized mechanism of pharmacologic action of DVE-MA-divalent cation complexes.

Structural Studies

It has been suggested [41] that the tetrahydrofuran structure for DVE-MA (I) is more in accord with experimental data from polymer



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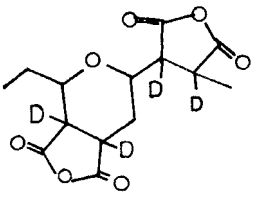
solution measurements on DVE-MA and its methyl ester than the originally proposed tetrahydropyran structure [5-9]. These arguments were believed to be supported by molecular model studies which revealed that the bulky carbomethoxy groups caused much more steric hindrance in the tetrahydropyran structure of DVE-MA than in the alternative tetrahydrofuran structure. However, potentiometric titration studies reported earlier [17] have been interpreted to mean that one of the carboxyl groups in the hydrolyzed form of DVE-MA is different from the other three, a structural feature which is not consistent with the tetrahydrofuran structure, but is consistent with the tetrahydropyran structure [5-9].

Recent work [42] which involved copolymerization studies with dideuteromaleic anhydride (II) and tetradeuteriodivinyl ether (III)



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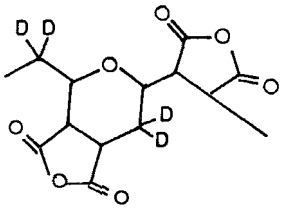
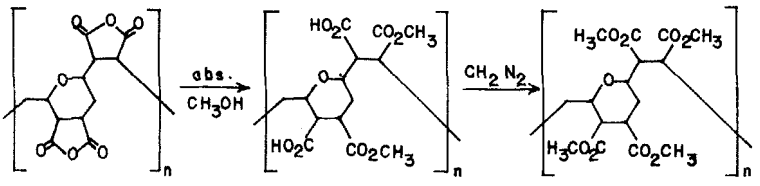


III

continue to support the tetrahydropyran structure. However, recently published data [43] strongly suggest that the ratio of five- to six-membered ring structure is solvent-dependent.

Extensive studies have been conducted on determination of molecular weight (MW) and molecular weight distribution (MWD) of DVE-MA prepared under various conditions [44]. Because of the problems of dealing with the polyelectrolyte effect during gel-permeation chromatographic studies, the copolymer was completely methylated [Eq. (3)] and the MW and MWD properties were obtained on the derivative of the original DVE-MA.



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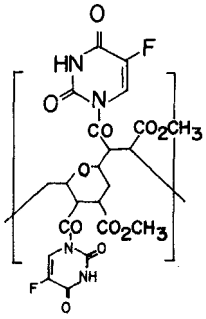
FUNCTIONAL DERIVATIVES OF DVE-MA

The DVE-MA copolymer owes much of its reactivity to the anhydride function, which can be easily converted to derivatives such as the mono and diester, the mono and diamide and the imide. Functional derivatives of DVE-MA may be prepared either via reaction of a suitable reactant with the preformed copolymer of appropriate molecular weight and molecular weight distribution or by introduction of the functional group into maleic anhydride followed by copolymerization with DVE. The latter approach has been taken by Umrigar, Ohashi, and Butler [45], who synthesized 1-(2-carbomethoxyacryloloyl)-5-fluorouracil (CMAFU) and copolymerized this monomer to produce the copolymer corresponding to the 5-fluorouracil-functionalized DVE-MA (IV).



IV

Copolymerization of (I) with styrene (St) and 2-chloroethyl vinyl ether (CEVE), as well as with divinyl ether (DVE), was achieved by carrying out the reactions in sealed tubes with cyclohexanone as the solvent and 2,2'-azobis-2-methylpropionitrile (AIBN) as the initiator. The results of these experiments are summarized in Table 1. An investigation of the properties of the copolymers revealed that all were hydrolyzed by water, resulting in the release of 5-FU, a property which should be of biological significance. The rate of release of 5-FU from copolymers of DMAFU with DVE, St, and CEVE, and from CMAFU was measured by using NMR and UV. It was shown that the copolymers released 5-FU much more slowly than CMAFU. It was also suggested that the hydrophobic character of the polymer is important for slow release of 5-FU.

The results of the evaluation of these copolymers by the Development Therapeutics Program, Division of Cancer Treatment, National

TABLE 1. Copolymerization Study of 1-(2-Carbomethoxyacrylolyl)-5-fluorouracil[a]

Comonomer	Molecular weight (M_n)[b]	Solubility[c,d]			Softening point (°C)
		Water	Methanol	aq. NaOH	
Styrene	5957	i	sp	vs	130-140
2-Chloroethyl vinyl ether	7296	i	sp	vs	125-130
Divinyl ether	5187	i	sp	vs	115-120

[a]Data of Umrigar et al. [45].
[b]Determined in acetone by vapor phase osmometry (VPO).
[c]Soluble with decomposition.
[d]i = insoluble, sp = sparingly soluble, vs = very soluble.

TABLE 2. Results of Evaluation of Polymers by Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute[a]

Dose mg/kg[b]	T/C (%)[c]		
	St/CMAFU (NSC 255081)	CEVE/CMAFU (NSC 255082)	DVE/CMAFU NSC 255083
400	94	129	112
200	192	165	189
	144	141	172
100	171	165	137
	127	127	140
50	133	123	125
25	115	117	105

[a]Data of Umrigar et al. [45].
[b]Intraperitoneal injection in CD_2F_1 male mice, 6 of 6 survivors after 5th day against P 388 lymphocytic leukemia.
[c]Ratio of test animal (T) to control (C) survival times.

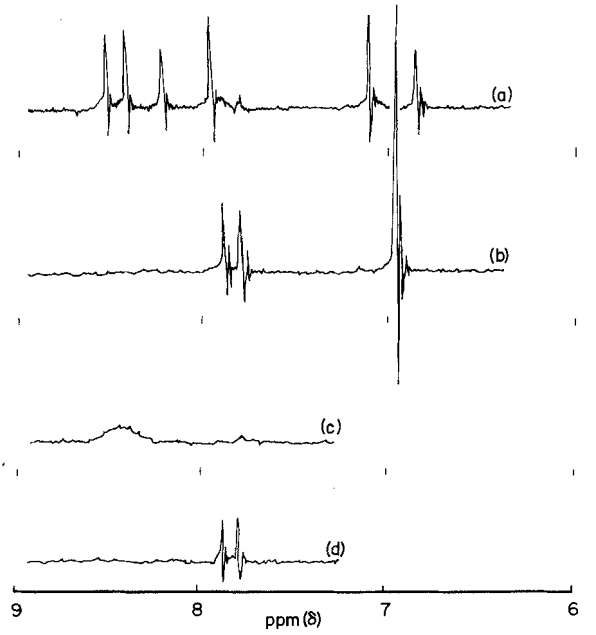


FIG. 1. NMR spectra of (a, b) CMAFU and (c, d) DVE/CMAFU before (a, c) and after (b, d) hydrolysis in acetone-d_6-D_2O (5:1). Reproduced from Umrigar et al. [45] with permission of the publishers.

Cancer Institute are shown in Table 2. The rate of release of 5-FU from the copolymer should have an important bearing on its effectiveness.

Rate of Hydrolysis by Use of NMR

The rates of hydrolysis of CMAFU and the copolymers were measured in acetone-d_6-D_2O (5:1 v/v). Figure 1 shows typical examples of NMR spectra before and after hydrolysis. Figure 2 shows the time-conversion curves. As shown in Fig. 2, CMAFU was hydrolyzed relatively fast, while the copolymers released 5-FU gradually. In the case of the copolymers almost half of the 5-FU units remained on the polymer chain even after two days. In the case of CMAFU, a first-order plot gave a straight line, and k_1 calculated was 3.67×10^{-5} liter/mole-sec.

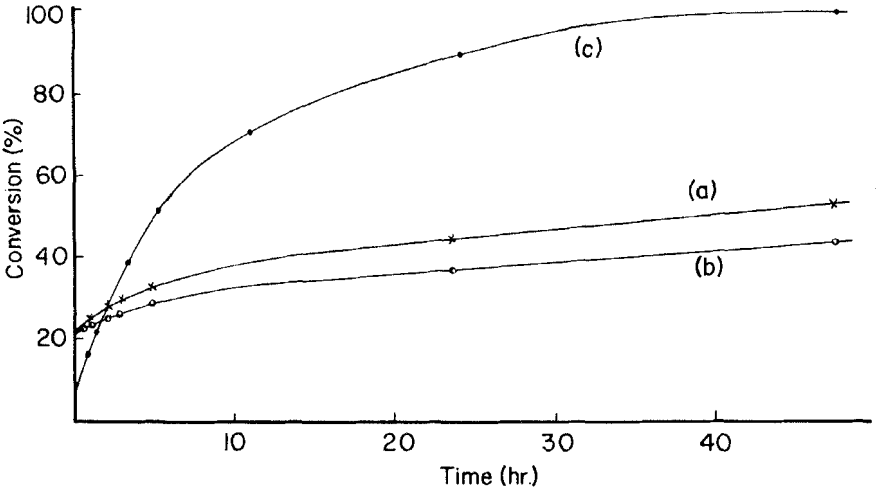


FIG. 2. Hydrolysis in acetone-d_6-D_2O (5:1): (a) DVE/CMAFU; (b) CEVE/CMAFU; (c) CMAFU. Reproduced from Umrigar et al. [45] with permission of the publishers.

Figure 2 shows that approximately 20% of 5-FU exists free just a few minutes after addition of D_2O. This phenomenon was explained by assuming that some portion of the 5-FU bonded to the polymer was very susceptible to hydrolysis and was released during the polymerization or purification procedure. Usually free 5-FU was removed by dissolving the sample in acetone and filtration, but the results indicated that some portion of the 5-FU was bonded to the polymer ionically and could not be removed. Under these conditions St/CMAFU was precipitated and the hydrolysis could not be followed.

Hydrolytic Rate by Use of UV

Figure 3 shows the UV spectra of 5-FU, CMAFU, and DVE/CMAFU. λ_{max} and ϵ in the region of 250-300 nm are shown in Table 3. In the case of CMAFU, the absorption at 300 nm was sufficiently strong that the rate of hydrolysis could be measured by following the decrease of absorbance. Figures 4 and 5 show the time-conversion curves and their first-order plots in the hydrolysis of CMAFU in water-dioxane. Table 4 shows their first-order rate constants. When the water content was lower, k_1 was in the range of 10^{-4}-10^{-5}, which agreed with the results obtained by using NMR. When the water content became higher,

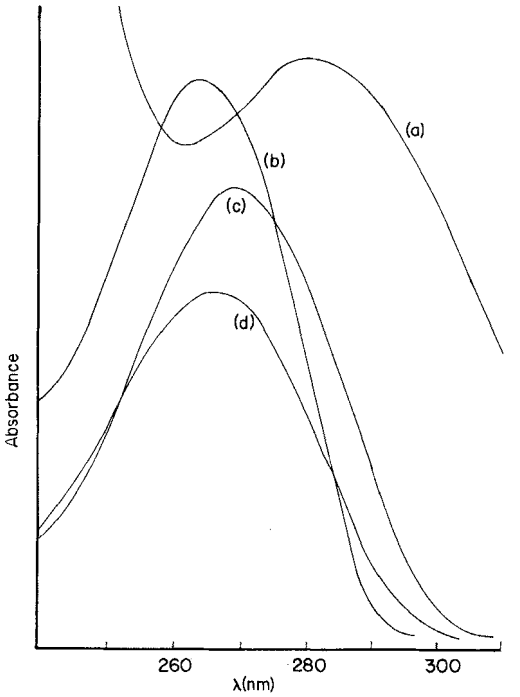


FIG. 3. UV spectra of 5-FU and 5-FU-containing compounds: (a) CMAFU in dioxane; (b) DVE/CMAFU in dioxane; (c) Floratur in H_2O; (d) 5-FU in H_2O. Reproduced from Umrigar et al. [45] with permission of the publishers.

TABLE 3. UV Spectra (λ_{max} and ϵ) of 5-FU and 5-FU-Containing Compounds

	Solvent[b]	λ_{max} (nm)	$\epsilon \times 10^{-3}$
5-FU	H_2O	265.6	7.19
CMAFU	Dioxane	275.5	8.00
DVE/CMAFU	Dioxane	264.3	8.13
CEVE/CMAFU	Dioxane	263.4	9.22
St/CMAFU	Dioxane	266.4	6.93

[a]Data of Umrigar et al. [45]
[b]Solvent for UV spectral measurements.

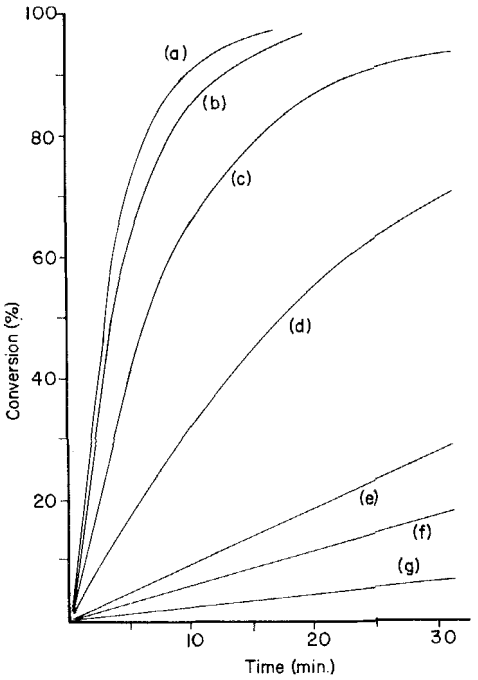


FIG. 4. Hydrolysis of CMAFU in dioxane-H_2O: (a) 1:10; (b) 1:5; (c) 1:2; (d) 1:1; (e) 2:1; (f) 3:1; (g) 5:1. Reproduced from Umrigar et al. [45] with permission of the publishers.

k_1 increased to reach almost the order of 10^{-2} in the absence of water. As the UV spectra of the copolymers were very close to that of 5-FU and also the solubility in water was very low, their hydrolysis in dioxane-water could not be investigated. Alternatively, hydrolysis in heterogeneous system was observed. The copolymers were dispersed in 0.5 M NaCl solution in order to decrease their solubility in water. Samples were removed periodically. After removing polymer by filtration, the filtrate was diluted to a predetermined amount and the amount of 5-FU released was determined by measuring its absorbance at 265.5 nm. The results are shown in Fig. 6. Under these conditions, CMAFU was hydrolyzed almost instantaneously. On the other hand, St/CMAFU and CEVE/CMAFU were resistant to hydrolysis and released 5-FU gradually. DVE/CMAFU was hydrolyzed faster than the other two copolymers, but much slower than CMAFU.

In this case also, the presence of free 5-FU was observed immediately after the start of the reaction which suggested that part of the

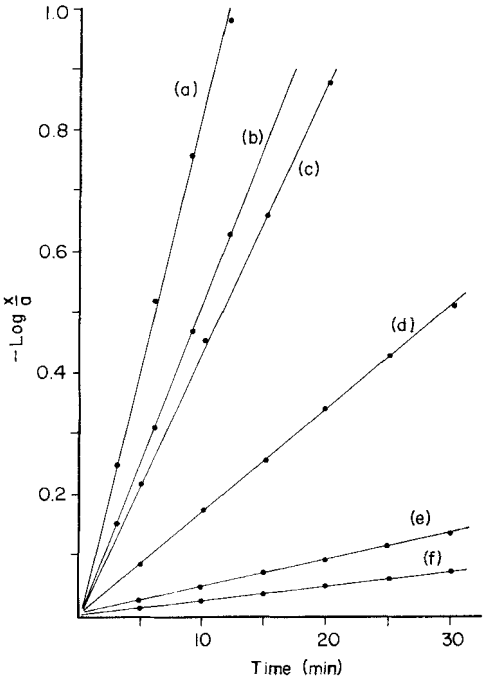


FIG. 5. Hydrolysis of CMAFU in dioxane-H_2O: (a) 1:10; (b) 1:5; (c) 1:2; (d) 1:1; (e) 2:1; (f) 3:1; (g) 5:1. The rate of decomposition is given by $-dx/dt = k_1x$, with $-\log(x/a) = k_1t$, where $x = [\text{CMAFU}]_{t=t}$, $a = [\text{CMAFU}]_{t=0}$, and k_1 is a first-order rate constant.

5-FU was released during the polymerization or purification procedure and could not be removed. In the reaction described above, distilled water or 0.5 M NaCl solution was used, and the pH of the reaction mixture was about 7 (Fig. 6). However, in order to study the mechanism of the hydrolysis, the hydrolysis in phosphate buffer (pH 6 and 8) was investigated. Under these conditions, CMAFU and DVE/CMAFU were solubilized and hydrolyzed almost instantaneously. However, St/CMAFU was hydrolyzed very slowly in spite of such severe conditions, and, even after 6 days, about 30-40% of 5-FU units remained on the polymer chain (Fig. 7). The effect of pH was not so remarkable which suggested that the reaction was catalyzed both by H^+ and OH^-.

From the data, it was shown that these copolymers were hydrolyzed much more slowly than CMAFU and released 5-FU very slowly.

TABLE 4. First-Order Rate Constants for Hydrolysis of CMAFU in H_2O-Dioxane at 25°C[a]

H_2O:Dioxane	k_1 (liter/mole-sec)
1:5	3.46×10^{-5}
1:3	1.03×10^{-4}
1:2	2.14×10^{-4}
1:1	6.54×10^{-4}
2:1	1.71×10^{-3}
5:1	3.22×10^{-3}
10:1	4.12×10^{-3}

[a]Data of Umrigar et al. [45].

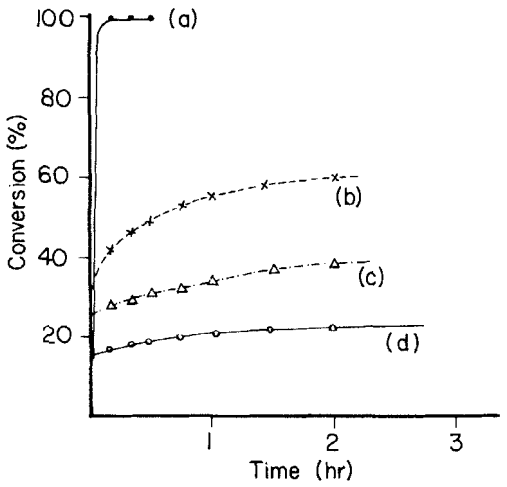


FIG. 6. Hydrolysis of 5-FU-containing compounds dispersed in 0.5 M NaCl (a) CMAFU, (b) DVE/CMAFU, (c) CEVE/CMAFU, (d) St/CMAFU. Reproduced from Umrigar et al. [45] with permission of the publishers.

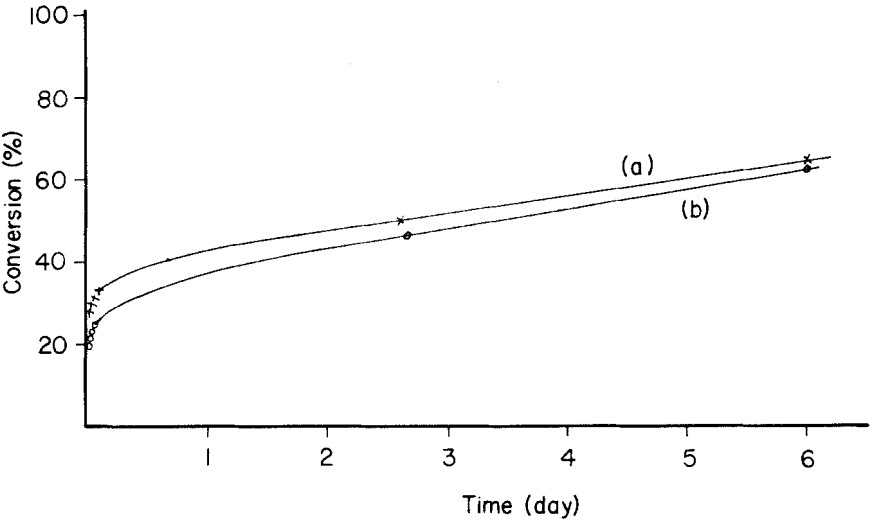


FIG. 7. Hydrolysis of St/CMAFU dispersed in 0.5 M phosphate buffer: (a) pH 6.00; (b) pH 8.00. Reproduced from Umrigar et al. [45] with permission of the publishers.

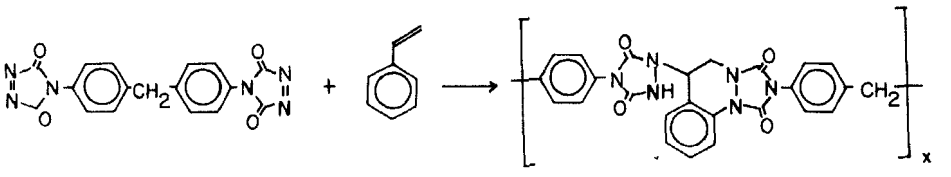
Especially St/CMAFU gave good results. DVE/CMAFU was more susceptible to hydrolysis. These results suggested that the hydrophobic character of the polymer as well as the strength of the bond with 5-FU is very important in decreasing the rate of hydrolysis.

Novel Polyanionic Structures

A novel type of polyanion has been developed in our laboratories during recent years. It has been shown that styrenes undergo a two-step reaction with N-substituted triazolinediones [46].

The resulting disubstituted urazole has been shown to have a pKa of $\alpha = 6$ in comparison to pKa's for carboxylic acids of $\alpha = 5$. Extension of the reaction to bistriazolinediones led to polymeric structures as shown in Eq. (4) and to the corresponding polyanions.

Polyurazole anions have also been prepared [Eqs. (5) and (6)] via the reaction of N-substituted triazolinediones with preformed polymers containing allylic hydrogens [47, 48]. This reaction is quite versatile, in that it occurs rapidly at room temperature and can be used to effect any degree of substitution desired.



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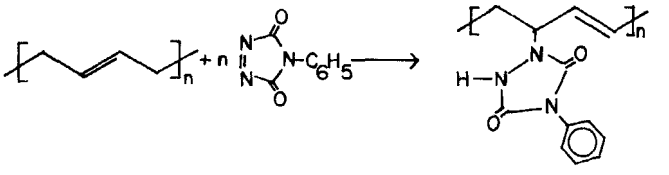

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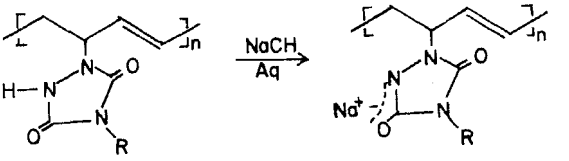
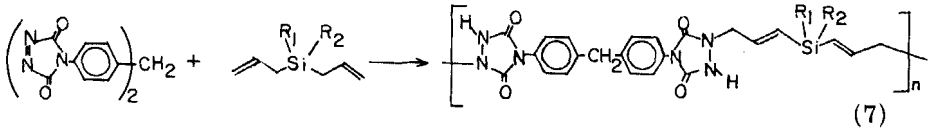


(6)



(7)

Another approach to synthesis of a variety of polyurazole anions is via the double-ene reaction with suitably selected nonconjugated dienes [Eq. (7)] [49]. There is considerable evidence in the case of the allyl silanes that the ene reaction involves the C–Si bond to some extent.

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