Hydrogels for Site-Specific Drug Delivery to the Colon: *In Vitro* and *in Vivo* Degradation

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Novel hydrogels based on *N,N*-dimethylacrylamide, *N*-t-butylacrylamide, and acrylic acid cross-linked with azoaromatic compounds of varying length and electron density of the azo bond were synthesized. The cross-links are degradable by microbial azoreductases present predominantly in the colon, and the gels appear to be suitable for colon-specific drug delivery. The degradability *in vitro* and *in vivo* was found to be related to the degree of swelling of the gels. The higher the degree of swelling, the higher the degradability. However, structural and electronic factors were also shown to influence reduction of azo bonds.

KEY WORDS: hydrogel; swelling; colonic drug delivery; degradation; azoreductase; activity.

INTRODUCTION

The colon can be used as a site for peptide drug delivery because of the low activity of proteolytic enzymes in the colon (1,2). Drug delivery to the colon can be obtained in several ways: (i) A controlled-release dosage form can release the drug after 5 hr, when the device can be assumed to have arrived in the colon (3), (ii) a pH-sensitive coating can protect the drug until arrival in the colon (4), and (iii) microbial enzymes present predominantly in the colon can be exploited in site-specific drug delivery to the colon (5–7). These methods were recently reviewed by Friend (8). The most suitable method relies on the unique ability of the colonic microbial flora to degrade otherwise stable compounds. For example, the degradation of azo compounds by microbial azoreductase activity was exploited in the preparation of low molecular weight (5) and polymeric (6,9) prodrugs containing azo bonds. A coating based on a branched polymer containing azoaromatic bonds, prepared by Saffran *et al.* (7), resulted in peptide drug release in the colon, where they were available for absorption.

The synthesis and characterization of novel hydrogels for site-specific peptide and protein drug delivery to the colon have been described earlier (10). The hydrogels contain acidic comonomers and enzymatically degradable azoaromatic cross-links. In the low pH of the stomach, the gels have a low degree of swelling, which protects the drug against degradation by digestive enzymes. As the gels pass down the gastrointestinal (GI) tract, the degree of swelling increases as a result of increased ionization and repulsion of charges on the gels with increasing pH. Upon arrival in the colon, the gels have reached a degree of swelling that makes the cross-links accessible to enzymes (azoreductase systems) or mediators (electron carriers). The cross-links are then degraded and the drug is released from the disintegrated gels. Many factors influence rates and mechanisms of degradation of hydrogels containing degradable cross-links. The degradation is dependent on the structure and length of the cross-linking agent and degree of swelling of the hydrogels (11). In this paper, the *in vitro* and *in vivo* degradation of hydrogels containing azoaromatic cross-links is described.

MATERIALS AND METHODS

Materials

4,4-Diaminoazobenzene and *N*-t-butylacrylamide (BuA; Polysciences) were recrystallized from ethanol and acetone, respectively. Acrylic acid (AA), *N,N*-dimethylacrylamide (DMA), dimethyl sulfoxide, methacryloyl chloride, and pyridine (all Aldrich) were distilled before use. Benzyl viologen (ICN Biochemicals), t-butylperoxyoctoate (Polysciences), 6-aminohexanoic acid, bromine, acetic acid (glacial), sodium acetate (anhydrous), tetrahydrofuran (THF), triethylamine (all Aldrich), α-D-glucose, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADP) (all Sigma) were used as obtained.

Synthesis of Cross-Linking Agents

4,4'-Di(methacryloylamino)azobenzene (DMAAB; Fig. 1a). 4,4'-Di(methacryloylamino)azobenzene was prepared by methacryloylation of 4,4'-diaminoazobenzene as described previously (10); m.p., 277–278°C.

*4,4'-Di(*N*-methacryloyl-6-aminohexanoylamino)azobenzene (DMCAAB; Fig 1b).* *N*-Methacryloyl-6-aminohexanoic acid was synthesized by methacryloylation of 6-aminohexanoic acid (12). *N*-Methacryloyl-6-aminohexanoic acid (0.03 mol, 6.0 g) was then dissolved in 20 ml THF, and triethylamine (0.03 mol, 4.2 ml) was added. After cooling to −15°C, isobutylchloroformate (0.03 mol, 3.9 ml) in 5 ml THF was added and the reaction mixture kept stirring at −15°C for 20 min. 4,4'-Diaminoazobenzene (0.015 mol, 3.2 g) was dissolved in 40 ml THF and added dropwise to the reaction mixture. The mixture was stirred overnight at room temperature, and the product filtered, washed with 1 *N* HCl, 5% sodium bicarbonate solution, and distilled water, dried, and recrystallized twice from ethanol. Yield, 4.33 g (50%); m.p., 212–214°C. Elemental analysis, found (calculated): C, 66.78 (66.88); H, 7.34 (7.37); N, 14.7 (14.62). The structure was verified with proton NMR.

3,3',5,5'-Tetrabromo-4,4,4',4'-tetra(methacryloylamino)azobenzene (BrDMAAB; Fig 1c). 4,4'-Diaminoazobenzene was brominated as described for the monosubstituted azobenzene (13,14). 4,4'-Diaminoazobenzene (0.025 mol, 5.3 g) was dissolved in 150 ml glacial acetic acid containing 12.3 g (0.15 mol) sodium acetate. The mixture was kept at 15°C and bromine (0.15 mol, 7.7 ml), in 232 ml glacial acetic acid,

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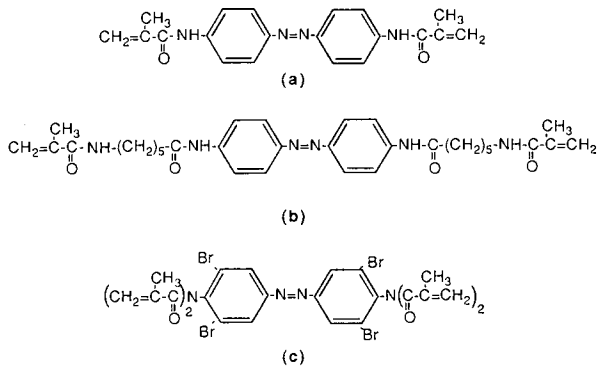

Fig. 1. Structure of cross-linking agents: (a) DMAAB; (b) DMCAAB; (c) BrDMAAB.

Table I. Composition of Hydrogels Based on 10% BuA, 40% AA, and 49.0–49.9% DMA (mol%)

Sample no.	Cross-linking agent	%[a]
1	DMAAB	0.1
2	DMAAB	0.2
3	DMAAB	0.5
4	DMAAB	1.0
5	DMCAAB	0.1
6	DMCAAB	0.2
7	DMCAAB	0.5
8	DMCAAB	1.0
9	BrDMAAB	0.2

[a] Percentage cross-linking agent in monomer mixture (mol%).

was added dropwise. After stirring overnight at room temperature, the precipitate was isolated, washed with water, and sodium thiosulfate, and dried. The tetrasubstituted 4,4′-diaminoazobenzene was isolated by dissolution in hot THF and the addition of water until turbidity occurred. The product was filtered, washed with THF and distilled water, and dried. Yield, 10.2 g (77.2%); m.p., 346–350°C (decomposes). Elemental analysis, found (calculated): C, 28.43 (27.31); H, 1.67 (1.53); N, 10.3 (10.61); Br, 58.59 (60.55). Proton NMR revealed that the bromide was placed in the 3,3′,5, and 5′ positions of the 4,4′-diaminoazobenzene. 3,3′,5,5′-Tetrabromo-4,4′-diaminoazobenzene (0.0018 mol, 0.9 g) was dissolved in 175 ml pyridine, and methacryloyl chloride (0.018 mol, 2.0 ml) was added dropwise under stirring at 70–75°C. After 5, 11, 22, and 28 hr an additional 1 ml (0.009 mol) of methacryloyl chloride was added. At 48 hr the reaction mixture was poured into 250 ml ice water and filtered, and the product recrystallized twice from ethanol. Yield, 0.78 g (55%). Elemental analysis, found (calculated): C, 42.21 (42.03); H, 3.04 (3.02); N, 6.94 (7.00); Br, 39.83 (39.95). Proton NMR spectrum verified that the compound was tetramethacryloylated.

Synthesis of Hydrogels

Terpolymers of acrylic acid, *N*-t-butylacrylamide, and *N,N*-dimethylacrylamide cross-linked with 4,4′-di(methacryloylamino)azobenzene, 4,4′-di(*N*-methacryloyl-6-aminohexanoylamino)azobenzene, or 3,3′,5,5′-tetrabromo-4,4,4′,4′-tetra(methacryloylamino)azobenzene were prepared as described previously (10). For composition of gels see Table I.

Preparation of Freeze-Dried Cecum Content

The cecum contents of male Sprague–Dawley rats (200 g) were isolated and suspended (1 g/10 ml) in nitrogenated distilled water. The suspension was filtered through glass wool and freeze-dried. The freeze-dried cecum content was stored at −20°C until use.

Preparation of Cell-Free Extract (CFE)

The procedure for preparation of CFE was modified from Brown *et al.* (9,15). The contents of cecum from male Sprague–Dawley rats (200 g) were isolated and suspended (1 g/10 ml) in 0.04 *M* potassium phosphate buffer, pH 7.4, previously bubbled with nitrogen. The mixture was sonicated (50 W) at 4°C for 20 min to disrupt the cells, filtered through glass wool, and centrifuged at 13,000 rpm (18,900*g*) for 30 min at 4°C (Beckman L2-65B ultracentrifuge). The supernatant containing the azoreductase activity was isolated and stored at −20°C until use.

Degradation of Cross-Linking Agents

The degradation mixture consisted (9) of 0.333 ml CFE/ml, 2.5×10^{-4} *M* NADP, 1.33×10^{-4} *M* benzyl viologen, 8.33×10^{-4} *M* glucose-6-phosphate, 0.167 ml ethanol/ml, and 1×10^{-5} *M* cross-linking agent in 0.04 *M* potassium phosphate buffer, pH 7.4. The mixture was bubbled with nitrogen for 5 min to obtain anaerobic conditions and 1 U/ml glucose-6-phosphate dehydrogenase was added to initiate the reaction. The vials were sealed and incubated in a thermostated water bath at 37°C. Samples were taken at 0, 10, 20, 40, and 60 min. The reaction was stopped by bubbling with oxygen and cooling in an ice bath.

The samples were analyzed by high-performance liquid chromatography (Waters, Millipore, MA). The column was a reverse-phase C-18 column and the mobile phase was 3:7 acetonitrile:water containing 0.1% trifluoroacetic acid for DMAAB and DMCAAB and 6:4 acetonitrile:water containing 0.1% trifluoroacetic acid for BrDMAAB. The samples were detected spectrophotometrically (UV detector, Waters) at their λ_{max}. DMAAB and DMCAAB were detected at 380 nm; BrDMAAB was detected at 330 nm. The retention times for DMAAB, DMCAAB, and BrDMAAB were 12, 12, and 7.5 min, respectively. Determinations were done in triplicate.

Degradation of Gels

In Vitro *Degradation.* Gel disks about 0.5 cm in diameter were swollen to equilibrium in 0.04 *M* potassium phosphate buffer, pH 7.4, and weighed. The gels were immersed in a cell suspension mixture consisting of 30 mg freeze-dried cecum content, 1.25×10^{-4} *M* benzyl viologen, 1.25 mg/ml α-D-glucose, and 4 ml 0.04 *M* potassium phosphate buffer, pH 7.4. The suspension was bubbled with nitrogen for 5 min to obtain anaerobic conditions, and the vials were closed and tightly sealed. The vials were incubated in a shaking water bath (50 strokes/min) at 37°C from 1 to 4 days. After degra-

dation the gels were recovered and washed in water and ethanol. The gels were swollen to equilibrium in 0.04 *M* potassium phosphate buffer, pH 7.4, and weighed. Then the gels were dried in a vacuum oven at 40–50°C for 2 days and the dry weight recorded.

In Vivo *Degradation.* Gels disks about 0.5 cm in diameter were preswollen in 0.04 *M* potassium phosphate buffer, pH 7.4, at 37°C. Each gel was placed in a nylon bag (1 × 1 cm, 0.5 mm in mesh size) and implanted in the cecum or stomach of male rats (Sprague–Dawley, 200 g) anesthetized with ether. The bag was secured with silk suture to the gut wall to prevent excretion of the gel. The rats recovered from anesthesia shortly after the surgery. The rats were allowed food and water ad libitum throughout the experiment. After 1 to 7 days the rats were sacrificed with an ether overdose and the gels retrieved from their guts. The gels were washed and swollen in 0.04 *M* potassium phosphate buffer, pH 7.4. The degradation was evaluated as described for *in vitro* degradation.

Data Analysis. The degradation was evaluated by an increase in swelling, $1/v_2$, where v_2 is the volume fraction of polymer in the swollen state calculated from Eq. (1):

$$v_2 = (m_2/d_2)/(m_2/d_2 + m_1/d_1) \quad (1)$$

where m_1 and m_2 are weights of water and polymer, respectively, and d_1 and d_2 are densities of water and polymer, respectively.

RESULTS AND DISCUSSION

Degradation of Cross-Linking Agents

The mechanism of azoreduction is not completely clear, but enough is known to be able to exploit this system in site-specific delivery of drugs to colon (16). It appears that the enzyme is intracellular and does not leak out of the cell, and NAD(P)H is needed as a cosubstrate. The increase in the reduction (cleavage) rate in the presence of electron carriers (16), as well as the observation (17) that the reduction of azo dyes increases with the onset of cell mortality, concurs with the hypothesis that there is an intracellular enzymatic component as well as extracellular reduction. Electron carriers function as exogeneous "electron shuttles" between azo compound and enzyme. The redox potential of the electron carrier is therefore of importance for the rate of reduction. The structure of the intermediate has not yet been proven; it is proposed that the reduction can proceed via a hydrazo intermediate (two-electron transfer) (18) or via an azo anion free radical (one-electron transfer) (19). The structure of the azo compound with regard to electron density (20) and stabilization by hydrogen bonding of the azo bond (21) influences the rate of reduction.

Since the cross-linking agents are not water soluble, ethanol had to be added to the enzyme mixture. However, it was shown in parallel experiments, using methyl orange as substrate, that the addition of ethanol to the incubation mixture decreased the activity of CFE by 40%, but the enzymatic system was still active (22).

Figure 2 shows the amount of cross-linking agent in the enzyme mixture as a function of time. BrDMAAB was degraded fastest, due to the decreased electron density of the azo bond, as bromide is electron withdrawing. As the electron density of the azobond decreased, attack of hydride ion or electron was faster and the rate of degradation increased. This correlates with results obtained by Zimmermann *et al.* (20). A Hammett plot of the logarithm of the relative rate of degradation was linearly dependent on the Hammett substituent constant of various substituents on derivatives of Orange II. DMCAAB was degraded more slowly than DMAAB, possibly because of the stabilization of the azo bond in the longer cross-linking agent by intermolecular hydrogen bonding between two molecules. This effect is not expected for the short cross-linking agent because of more steric hindrance. It was previously suggested that stabilization of the azo bond by hydrogen bonding, as in Red 2G, would decrease the rate of reduction (21).

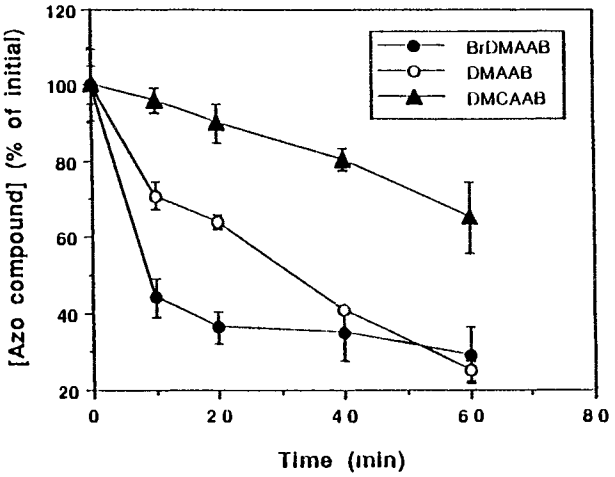

Fig. 2. Degradation of cross-linking agents. Concentration as percentage of initial concentration as a function of time of incubation. The degradation mixture consisted of 0.333 ml CFE/ml, 2.5×10^{-4} *M* NADP, 1.33×10^{-4} *M* benzyl viologen, 8.33×10^{-4} *M* glucose-6-phosphate, 0.167 ml ethanol/ml, 1 U/ml glucose-6-phosphate dehydrogenase, and 1×10^{-5} *M* cross-linking agent in 0.04 *M* potassium phosphate buffer, pH 7.4.

Degradation of Gels

Figures 3 and 4 show the equilibrium degree of swelling plotted as $1/v_2$ versus the time of incubation for gels containing DMAAB and DMCAAB as cross-linking agents, respectively. As the time of incubation increased the degree of swelling increased due to a decrease in the cross-linking density since the cross-links were cleaved. Gels with different cross-linking densities were investigated. As the cross-linking density decreased, the degradability increased. This can be explained by increased accessibility of the cross-links and fewer cross-links to be cleaved with decreased cross-linking density. Two gels with the same cross-linking density but different thicknesses, 0.5 and 1 mm, were degraded (Fig. 3). The thin gel degraded faster than the thick gel and disintegrated after 3 days of incubation.

Gels containing the longer cross-linker (DMCAAB) with the same cross-linking density (22) as gels containing DMAAB show more swelling, and they appear to degrade faster. This result can be attributed to an increased degree of swelling of these gels and increased accessibility to the azo

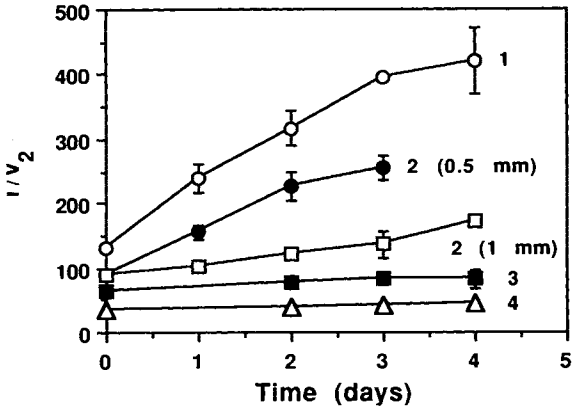

Fig. 3. *In vitro* degradation of gels cross-linked with DMAAB. Equilibrium degree of swelling ($1/v_2$) as a function of time of incubation. For composition of gels see Table I. Gel disks about 0.5 cm in diameter swollen to equilibrium in 0.04 *M* potassium phosphate buffer, pH 7.4, were immersed in a cell suspension mixture consisting of 30 mg freeze-dried cecum content, 1.25×10^{-4} *M* benzyl viologen, 1.25 mg/ml α-D-glucose, and 4 ml 0.04 *M* potassium phosphate buffer, pH 7.4.

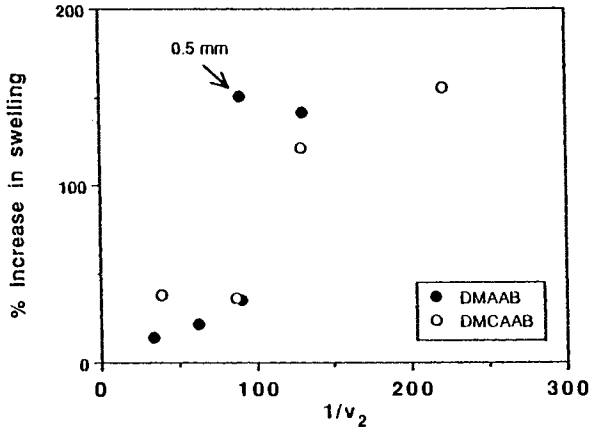

Fig. 5. *In vitro* degradation of gels cross-linked with DMAAB or DMCAAB. Percentage increase in swelling after incubation for 2 days as a function of the initial equilibrium degree of swelling. Gel disks about 0.5 cm in diameter swollen to equilibrium in 0.04 *M* potassium phosphate buffer, pH 7.4, were immersed in a cell suspension mixture consisting of 30 mg freeze-dried cecum content, 1.25×10^{-4} *M* benzyl viologen, 1.25 mg/ml α-D-glucose, and 4 ml 0.04 *M* potassium phosphate buffer, pH 7.4.

bonds for enzymes or mediators. For DMCAAB in solution the azo bond appeared to be stabilized against reduction by intermolecular hydrogen bonding. When DMCAAB was incorporated into a gel, this effect was not observed. Since DMCAAB was restrained as cross-links in the gel it did not possess the flexibility to form hydrogen bonds.

Figure 5 illustrates the dependence of the percentage increase in the equilibrium degree of swelling after degradation. This confirms that degradability is related to equilibrium degree of swelling; as the degree of swelling increases, the degradability increases. These results correlate with results found by Ulbrich *et al.* (11) and Šubr *et al.* (23). They prepared gels based on *N*-(2-hydroxypropyl)methacrylamide containing oligopeptide sequences in the cross-links. The degradation was investigated using chymotrypsin (11,23) and isolated lysosomal enzymes from rat liver (23) as model enzymes. The rate of degradation was found to be dependent on the structure and length of the peptide sequence, network density, and equilibrium degree of swelling. As the cross-linking density increased, steric hindrance prevented the enzyme–substrate complex from being formed and the rate of degradation decreased.

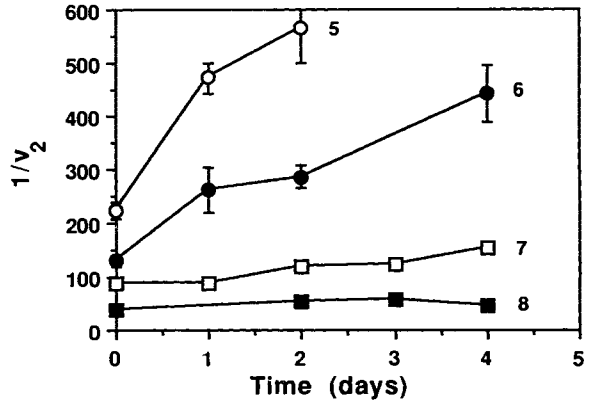

Fig. 4. *In vitro* degradation of gels cross-linked with DMCAAB. Equilibrium degree of swelling ($1/v_2$) as a function of time of incubation. For composition of gels see Table I. Gel disks about 0.5 cm in diameter swollen to equilibrium in 0.04 *M* potassium phosphate buffer, pH 7.4, were immersed in a cell suspension mixture consisting of 30 mg freeze-dried cecum content, 1.25×10^{-4} *M* benzyl viologen, 1.25 mg/ml α-D-glucose, and 4 ml 0.04 *M* potassium phosphate buffer, pH 7.4.

Gels containing the brominated cross-linker BrDMAAB did not increase in equilibrium degree of swelling after incubation. It was shown from degradation of cross-linking agents alone that the brominated cross-linking agent is degraded faster than the other cross-linking agents. However, since BrDMAAB is tetrafunctional, there is the possibility of forming cross-links that do not include the azo bond. In that case reduction of the azo bond would not result in cleavage of the cross-links.

Figure 6 shows the equilibrium degree of swelling as $1/v_2$ for gels implanted in rat stomach or cecum versus the time of implantation. Only gels implanted in the cecum increased in equilibrium degree of swelling, indicating that only these gels were degraded. Gels implanted in the stomach did not change in equilibrium degree of swelling. This confirms that only the cecum, and not the stomach, contains azoreductase activity.

An increase in swelling after degradation indicates bulk degradation. This is often observed for hydrophilic materials, such as hydrogels (24). Degradation of gels could be followed macroscopically by a change in color (7), since the azo compound was yellow, and as cross-links degraded, the color changed from yellow to colorless. Gels with a low degree of cross-linking became colorless faster than gels with a higher degree. Gels with a higher degree of cross-linking showed a colorless edge with a yellow core. Enzymes and cofactors are able to diffuse into the gels. However, evaluating the color change during degradation, it appears that the degradation in the case of less swollen gels proceeds from the surface. As the gels partially degrade and swelling

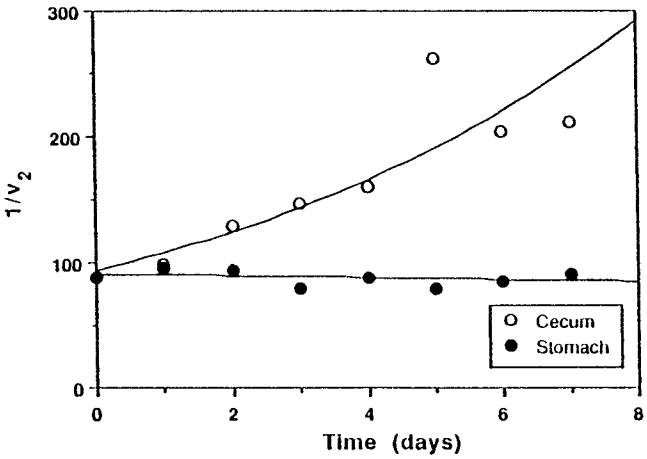

Fig. 6. *In vivo* degradation of gel (sample 2). Equilibrium degree of swelling ($1/v_2$) as a function of time of implantation in rat stomach or cecum. For composition of gel see Table 1. Gel disks about 0.5 cm in diameter were placed in a nylon bag and implanted in the cecum or stomach of male rats (Sprague–Dawley, 200 g) anesthetized with ether. After 1 to 7 days the rats were sacrificed with an ether overdose, and the gels retrieved from their guts. The gels were washed and swollen in 0.04 *M* potassium phosphate buffer, pH 7.4, and the degradation was evaluated.

increases, degradation also proceeds by bulk degradation. Poly(1-vinyl-2-pyrrolidone) hydrogels containing albumin cross-links were prepared by Shalaby and Park (25). The albumin cross-links were degraded by pepsin in the stomach, resulting in an increased degree of swelling, prolonging the retention time and release of drug. Degradation of these gels was also found to take place by both surface erosion and bulk degradation.

The gels did not totally degrade and disintegrate under the experimental conditions used. Gels containing similar azoaromatic cross-links but synthesized by cross-linking of polymeric precursors (1) appeared to be degraded faster than the present gels synthesized by cross-linking copolymerization. Several factors could contribute to this phenomenon. Side reactions during copolymerization could result in nondegradable cross-links, but more likely, differences in the structure of the networks, such as spacing of cross-links along the polymer backbone, could influence the rate of degradation. Another factor is that the molecular weight of the polymer main chain is much higher for gels synthesized by cross-linking copolymerization than for gels synthesized by cross-linking of polymeric precursors; this would result in a more entangled network for copolymerized gels.

CONCLUSIONS

The *in vitro* and *in vivo* degradability of gels containing azoaromatic cross-links was evaluated. The degradability was related to the structure of the cross-linking agent and equilibrium degree of swelling of the gels. As the cross-linking density increased, the degradability decreased. Gels containing a longer cross-linker appeared to be degraded faster than gels containing the shorter cross-linker. Degradation of the gels appeared to proceed in two steps. At first, the degradation seemed to proceed from the surface; as the cross-links were partially degraded, the degree of swelling increased, allowing enzymes and mediators to diffuse into the gel. Consequently, the degradation took place by bulk degradation as well.

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