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Influence of the composition of in-vitro azo-reducing systems on the degradation kinetics of the model compound amaranth

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Abstract

The purpose of this study was to investigate the influence of the composition of in-vitro azo-reducing systems on the degradation kinetics of the model compound amaranth. The degradation kinetics of amaranth were determined under anaerobic conditions both in rat caecal content (ex-vivo) and in a variety of in-vitro degradation media derived from rat caecal content. It was observed that the reducing activity was highly dependent on the preparation method and composition of the degradation medium. In pure rat caecal content, the degradation of amaranth was apparent first order ($k = 0.044 \pm 0.002\ min^{-1}$), while dilution of the rat caecal content resulted in an apparent zero-order degradation. Both apparent zero- and first-order degradations were also observed in media made up of diluted rat caecal content to which cofactors such as NADP, D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase and Bz were added. This study demonstrates that in-vitro azo-reducing kinetics are dependent on the composition and mode of preparation of the in-vitro media used. This has to be taken into account when evaluating the degradability of azo-aromatic drug delivery systems in-vitro.

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Introduction

Azo-containing colon-specific drug delivery systems have been explored in order to release an orally taken drug in the colon. Release of the drug is supposed to take place after reduction, and thus degradation, of the azo bonds by the colonic microflora. In order to correctly evaluate the degradation of a colon-specific drug delivery system, an adequate screening method for its degradation is necessary. In-vitro screening systems are preferred to in-vivo evaluation as fewer animals are needed and sample handling is easier.

Several in-vitro evaluation media have been described in the literature (Brown et al 1983; Kopecekova & Kopecek 1990; Brondsted & Kopecek 1992; Kopecekova et al 1994; Van den Mooter et al 1994; Yeh et al 1994; Ghandehari et al 1997; Kakoulides et al 1998), consisting of a suspension or a cell-free extract of rat caecal content, to which in some cases different cofactors were added and which was prepared under anaerobic conditions. Other researchers (Saffran et al 1986; Kimura et al 1992; Shantha et al 1995; Ueda et al 1996) used a suspension of human faeces to which cofactors were added in some cases. Alternatively, an in-vitro chemical reductive medium consisting of a solution of sodium sulfide and cysteine has been described (Schacht et al 1996).

The reducing activity of these systems has mostly been verified semi-quantitatively with a model azo compound. However, none of these media has ever been correlated to a real in-vivo situation. The aim of the present study was therefore to investigate the influence of the composition and the preparation mode of in-vitro reducing systems on the degradation kinetics of a water-soluble, frequently used model azo compound. This was done by comparing the in-vitro degradation kinetics of the model compound amaranth to those in a so-called ex-vivo situation with rat caecal content. The ex-vivo situation is supposed to be representative for the in-vivo situation, and is used because in-vivo testing of the degradation kinetics is impossible from a practical point of view.

In a first set of experiments the degradation kinetics of amaranth in rat caecal content (ex-vivo) under anaerobic conditions were investigated. Subsequently, several preparation parameters of an in-vitro rat caecal content medium were varied in order to evaluate their influence on the degradation kinetics of amaranth.

Materials and Methods

Composition of stock solutions

All 0.05 M phosphate buffer solutions of pH 6.8 were sterilized before use by autoclaving at 120°C for 20 min. Bz solution = 2.24 mM solution of benzylviologen dichloride (Sigma, St Louis, MO) in 0.05 M phosphate buffer pH 6.8. NADP solution = 4 mM solution of β-nicotinamide adenine dinucleotide phosphate (sodium salt; Sigma) in 0.05 M phosphate buffer pH 6.8. G6P solution = 13.6 mM solution of D-glucose-6-phosphate (Sigma) in 0.05 M phosphate buffer pH 6.8. DH solution = 16 U mL^{-1} solution of glucose-6-phosphate dehydrogenase (type V; Sigma) in 0.05 M phosphate buffer pH 6.8. Standard amaranth solution = 41.5 mM solution of amaranth (Friedrich-Karl Marcus, Geesthacht-Elbe, Germany). This solution was sterilized by autoclaving at 120°C for 20 min. Amaranth solution $1/x$: a $1/x$ dilution of standard amaranth solution in 0.05 M phosphate buffer pH 6.8.

Animal experiments

All experiments with animals carried out in this study adhered to *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985).

Preparation of the rat caecum suspension

Male Wistar rats ($\pm$300 g) were killed by injecting them with a lethal dose of pentobarbital (Nembutal, 200 mg kg^{-1}). The caecum was ligated, removed and its weight was determined, after which it was immediately transferred to the anaerobic workstation (Compact anaerobic workstation, Don Whitley Scientific, UK), which has an atmosphere of Anaero 10 (consisting of 10% hydrogen, 10% carbon dioxide and 80% nitrogen; Air Liquide, Liège, Belgium). After removal of its content, the caecum was weighed again in order to determine the weight of the content. The caecal content was subsequently suspended in 0.05 M phosphate buffer of pH 6.8 (10 mL of buffer per gram of caecal content), and filtered through glass wool to remove debris.

Degradation study of amaranth in-vitro

Preparation of the media

Influence of centrifugation (M1) The suspension of rat caecal content was purged for 15 min with Anaero 10, after which the suspension was covered with aluminium foil, removed from the workstation and sonicated (MSE sonicator, Analis, Namur, Belgium) for 20 min while cooling in ice. The sonicated suspension was then centrifuged at 4°C for 30 min (RC5C Centrifuge, SS-34 Rotor, Sorvall Instruments, USA) at 47808 *g* (a), or 478 *g* (b) or not centrifuged (c). The supernatant or suspension was then transferred to the anaerobic workstation and 3.75 mL of Bz solution, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution were added to 60 mL of the suspension or supernatant. This mixture was purged again with Anaero 10 for 15 min.

Influence of cofactors (M2) The suspension of rat caecal content was purged for 15 min with Anaero 10, after which the suspension was covered with aluminium foil, removed from the workstation and sonicated for 20 min while cooling in ice. The suspension was transferred to the anaerobic workstation and to 60 mL of the suspension was added 3.75 mL of Bz solution, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution (a). To another 60 mL of the suspension 15 mL of phosphate buffer pH 6.8 was added (b). The mixtures were purged again with Anaero 10 for 15 min.

Influence of sonication (M3) The suspension of rat caecal content was purged for 15 min with Anaero 10, after which it was covered with aluminium foil and

removed from the workstation. Half of the suspension was sonicated (a) for 20 min while cooling in ice. The other half was not sonicated (b). Both suspensions were transferred to the anaerobic workstation and to 60 mL of each suspension was added 3.75 mL of Bz solution, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution. These mixtures were purged again with Anaero 10 for 15 min.

Influence of Bz concentration (M4) To 60 mL of the suspension of rat caecum 3.75 mL of Bz solution, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution (a) was added. To another 60 mL of the suspension 2.5 mL of Bz solution, 1.25 mL of phosphate buffer, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution (b) was added, and to a final 60 mL of the suspension 1.25 mL of Bz solution, 2.5 mL of phosphate buffer, 3.75 mL of NADP solution, 3.75 mL of G6P solution and 3.75 mL of DH solution (c) was added. These mixtures were purged with Anaero 10 for 15 min.

Degradation study

One millilitre of amaranth solution 1/8 was added to 15 mL of the degradation medium (M1 to M4). At predetermined time intervals samples of 1 mL were taken from the reaction medium and added to a test-tube containing 2 mL of octanol (Riedel-de Haën, Germany) and 1 mL of concentrated hydrochloric acid (Merck, Germany), and the test-tube immediately shaken vigorously in order to allow the amaranth to be extracted to the octanol layer. After finishing the kinetic experiments in the anaerobic workstation, all the test-tubes were removed and centrifuged (ALC PJ 180R centrifuge, Milan, Italy) at 2650 *g* for 1 min at 4°C. One millilitre of the octanol layer from each test-tube was added to another test-tube containing 3 mL of a solution of 0.5 M Na_3PO_4 (Acros, Geel, Belgium). This test-tube was shaken by vortexing for 20 s in order to extract the amaranth to the phosphate layer, and centrifuged at 2650 *g* for 1 min at 4°C. The amaranth concentration was determined spectrophotometrically by measuring the absorbance of the phosphate layer at 499 nm (Uvikon 810P spectrophotometer, Kontron Instruments, Switzerland).

For each experiment a new calibration curve was made. For this purpose a series of dilutions (1–1/512) in 0.05 M phosphate buffer at pH 6.8 was made starting from amaranth solution 1/8. 0.1 mL of each dilution was added to a test-tube containing 3.2 mL of octanol and 1.6 mL of concentrated hydrochloric acid. After shaking the test-tube, 1.5 mL of the degradation medium was added and the test-tube was shaken again and centrifuged at 2650 *g* for 1 min at 4°C. One millilitre of the octanol layer was added to another test-tube containing 3 mL of a solution of 0.5 M Na_3PO_4. This test-tube was shaken by vortexing for 20 s in order to extract the amaranth to the phosphate layer, and centrifuged at 2650 *g* for 1 min at 4°C. The absorbance of the phosphate layer was determined spectrophotometrically at 499 nm.

Degradation study of amaranth ex-vivo

Rats were killed by injecting them with a lethal dose of pentobarbital (Nembutal, 200 mg kg^{-1}). Under anaerobic conditions the caecum was removed. For experiment EV1, the caecal content was used as it was. For the other experiments the caecal content was diluted with phosphate buffer 0.05 M pH 6.8: 2/3 for EV2, 1/3 for EV3. The content or the dilutions were transferred in tared hermetically closable plastic tubes so that every tube contained about 500 mg of rat caecal content for EV1 and 500 mg of a given dilution of caecal content for the other experiments. To every tube was added 0.1 μL of amaranth solution per milligram of caecal content or of the dilution: for EV1 standard amaranth solution was used, for EV2 amaranth solution 2/3, for EV3 amaranth solution 1/3. After predetermined incubation times 5 mL of concentrated hydrochloric acid and 10 mL of octanol were added and the tube was closed and shaken immediately for 20 s. After finishing the kinetic experiments in the anaerobic workstation, all the tubes were removed and centrifuged at 2650 *g* for 1 min at 4°C. One millilitre of the octanol layer was removed from the tube and transferred to a tube containing 3 mL of a solution of 0.5 M Na_3PO_4. This test-tube was vortexed for 20 s in order to extract the amaranth to the phosphate layer, followed by centrifugation at 2650 *g* for 1 min at 4°C. The amaranth concentration was determined spectrophotometrically by measuring the absorbance of the phosphate layer at 517 nm.

For each experiment a new calibration curve was made. For this purpose a series of dilutions (1–1/512) in 0.05 M phosphate buffer at pH 6.8 was made starting from standard amaranth solution for EV1, from amaranth solution 2/3 for EV2 and from amaranth solution 1/3 for EV3. Fifty microlitres of each dilution was added to a test-tube containing 10 mL of octanol, 5 mL of concentrated hydrochloric acid and about 500 mg of rat caecal content. The tube was shaken vigorously before and after addition of the dilution. One millilitre

of the octanol layer was removed from the test-tube and transferred to a test-tube containing 3 mL of a solution of 0.5 M Na_3PO_4. This test-tube was shaken by vortexing for 20 s in order to extract the amaranth to the phosphate layer, and centrifuged at 2650 *g* for 1 min at 4°C. The absorbance of the phosphate layer was determined spectrophotometrically at 517 nm.

Kinetic analysis

Data points were fitted using linear and non-linear curve-fitting procedures provided in the Origin 5.0 software (Microcal Software Inc., USA) in order to assign zero- or first-order degradation kinetics. The goodness of fit of a data set to a zero- or first-order degradation model was evaluated by comparing the coefficient of determination (COD) at the 95% confidence interval. The concentration at time zero was set at a fixed value during the fitting procedure.

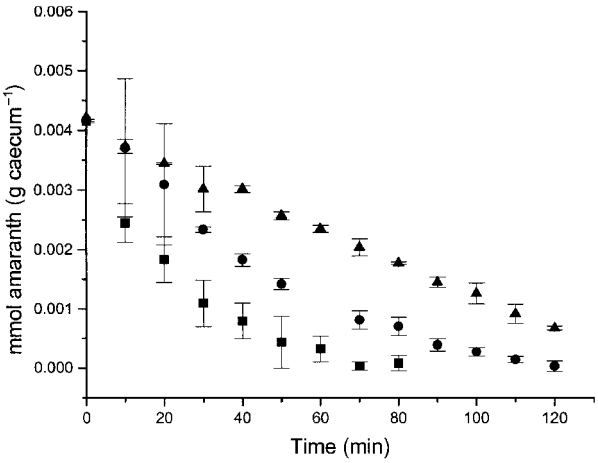

Figure 1 Ex-vivo degradation of amaranth in pure rat caecal content (■), in 2/3 dilution of rat caecal content in phosphate buffer 0.05 M pH 6.8 (●) and in 1/3 dilution of rat caecal content in phosphate buffer 0.05 M pH 6.8 (▲) (n = 3, mean±s.d.).

Results and Discussion

Reproducible spectrophotometrical determination of the amaranth concentration in the degradation medium was only possible after separation of amaranth from both the caecal content and the cofactors. Indeed, direct spectrophotometrical determination of the amaranth concentration in the degradation medium is impossible because of two reasons, the first being the turbidity of the degradation medium due to components of the rat caecal content. Another hindering factor is the absorbance by components of the rat caecal content and cofactors at the same wavelength area as amaranth, leading to an uncontrollable variation during the experiment, resulting in non-reproducible absorbance of the blank sample. Separation of amaranth from rat caecal content components and cofactors can be achieved as described above by extracting the amaranth from the degradation medium with octanol, followed by extraction with sodium phosphate. An additional advantage of this separation procedure is that it will stop the reduction of the model compound after a predetermined reaction time since it separates amaranth completely from the reducing substances in the degradation medium.

Since quantitative measurements of the degradation of amaranth in-vivo are extremely difficult to perform, it was decided to compare the in-vitro degradation kinetics to ex-vivo degradation kinetics, and the experiments were set up in such a way that it can be assumed that there is a maximal similarity with the in-vivo situation. For that purpose, the caecum was rapidly removed from the rat under strict anaerobic conditions in order to prevent any contact of the caecal content with oxygen, and the amaranth was added as a concentrated solution in order to minimize the dilution of the caecal content.

In order to be able to correctly compare the degradation kinetics of the pure rat caecal content (EV1) to those with diluted rat caecal content (EV2, EV3), the ratio of amaranth versus rat caecal content was kept constant over all the EV experiments (4.15×10^{-3} mmol per gram caecum content). The results of the degradation kinetic studies of amaranth ex-vivo with pure caecal content (EV1) and with diluted caecal content (EV2, EV3) are depicted in Figure 1. A control experiment in which amaranth was incubated in phosphate buffer (0.05 M, pH 6.8) showed that the remaining amaranth concentration was 98.3 (±0.7) and 101.4 (±1.9)% after 1 and 2 h, respectively. The degradation rate in rat caecal content seemed to be dependent on the residual amount of amaranth (apparent first-order degradation: $C_t = C_0 \times \exp(-kt)$, in which C_t is the concentration of amaranth at time t, C_0 is the starting concentration, k is the rate constant and t represents the time) if pure caecal content (EV1) or only slightly diluted (2/3) caecal content (EV2) was used. However, apparent zero-order (linear) degradation ($C_t = C_0 - kt$) was observed if more diluted (1/3) rat caecal content was used (EV3). Table 1 gives an overview of the degradation rate constants (k) and the half-lives of amaranth in the different ex-vivo degradation media. Both linear and non-linear degradation of azo compounds by anaerobic

Table 1 Degradation constants and half-lives of amaranth in EV1-3.

Experiment	k (s.d.)	$t_{1/2}$ (min)
EV1	0.0440 (0.0020) min^{-1}	16.16
EV2	0.0230 (0.0010) min^{-1}	26.40
EV3	2.8400×10^{-5}	42.73
	(6.2900×10^{-7}) mol (g caecum^{-1}) per min	

s.d. = standard deviation (n = 3).

Table 2 Degradation constants (k) and half-lives of amaranth in M1–4.

Experiment	k (s.d.)	$t_{1/2}$ (min)
M1		
No centrifugation	0.0740 (0.0065) min^{-1}	13.82
478 *g*	Mixed mechanism	22.18
47808 *g*	0.0049 (0.0002) mmol L^{-1} per min	31.01
M2		
With cofactors	0.0835 (0.0082) min^{-1}	12.40
Without cofactors	0.0020 (0.0001) mmol L^{-1} per min	72.21
M3		
Sonicated	0.0938 (0.0055) min^{-1}	10.79
Not sonicated	0.1728 (0.0106) min^{-1}	6.58
M4		
0.037 mM Bz	0.0100 (0.0004) mmol L^{-1} per min	15.14
0.075 mM Bz	Mixed mechanism	11.86
0.112 mM Bz	0.1084 (0.0083) min^{-1}	10.02

s.d. = standard deviation (n = 3).

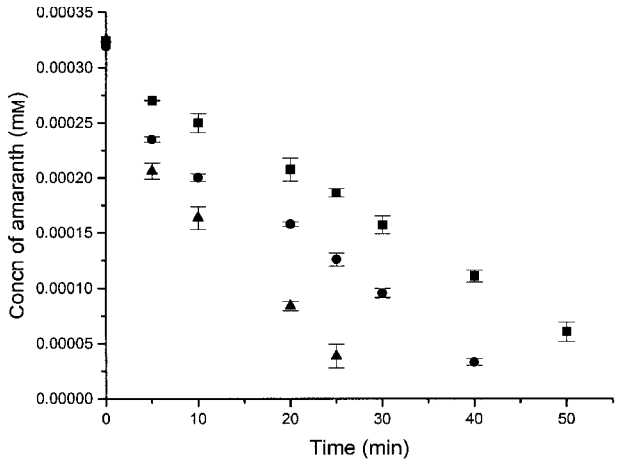

Figure 2 Influence of centrifugation speed on amaranth degradation: ▲ = no centrifugation; ● = centrifugation at 478 *g* for 30 min; ■ = centrifugation at 47808 *g* for 30 min (n = 3, mean±s.d.).

bacteria have already been described in the literature. Van den Mooter et al (1994) reported a linear degradation of tartrazine in a cell-free extract of rat caecal content to which several cofactors were added. Zero-order degradation of azo bonds has also been observed with cultures of *Streptococcus faecalis* by Walker et al (1971), of *Proteus vulgaris* by Dubin & Wright (1975), of *Bacteroides fragilis* by Bragger et al (1997), and of *Clostridium perfrigens* by Semdé et al (1998). On the other hand, the degradation rate of azo dyes by *Bacillus cereus* seemed to be dependent on the azo concentration in a study by Wuhrmann et al (1980). The observations in the present study illustrate that the degradation kinetics of the azo compound under investigation are dependent on the quantitative composition of the degradation medium.

In order to evaluate the influence of the composition of a degradation medium, several in-vitro media were prepared and the degradation kinetics of amaranth in these media were determined. Table 2 gives an overview of the rate constants (k) and half-lives of amaranth in the different in-vitro media.

It is generally accepted that azo reduction by anaerobic bacteria takes place by a chain of reduction reactions in which an intracellular NADPH-dependent flavoprotein reduces an electron mediator such as flavine mononucleotide or flavine adenine dinucleotide, after which the electron carrier reduces the azo bond (Walker et al 1971). For this reason NADP, G6P and DH are added in the in-vitro experiments as an NADPH-generating system, and Bz is added as additional electron mediator.

In experiments using M1 it was shown that part of the degrading activity was lost if the medium was centrifuged before use and that the activity decreased with an increasing centrifugation speed (Figure 2). It was observed that degradation of amaranth follows apparent first-order kinetics if no centrifugation is applied, while centrifugation at 47808 *g* shifts the kinetics to a zero-order mechanism. At 478 *g* no significant difference was observed in COD if the data were fitted to a zero- or first-order model, and this suggests that the system is in a transitory state. Indeed, taking a closer look at this data showed that initially a first-order process dominates, while from 20 min on, a zero-order process rules. Since part of the azo-reducing activity is removed by centrifugation, it could be assumed that some bacterial cells survive sonication and are removed during centrifugation. Another, more likely, assumption is that at

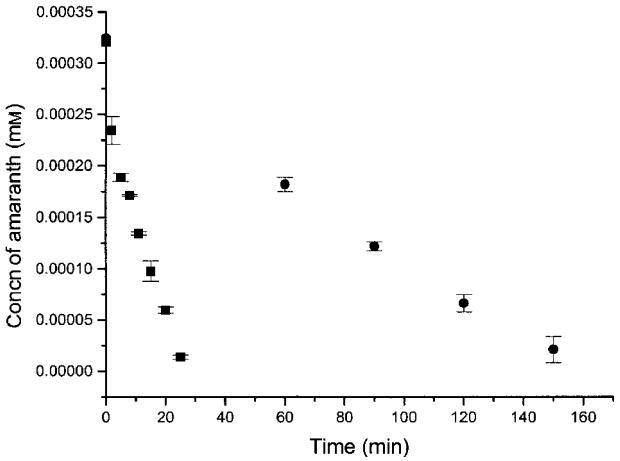

Figure 3 Influence of addition of cofactors on the degradation of amaranth: ■ = addition of cofactors; ● = no addition of cofactors (n = 3, mean±s.d.).

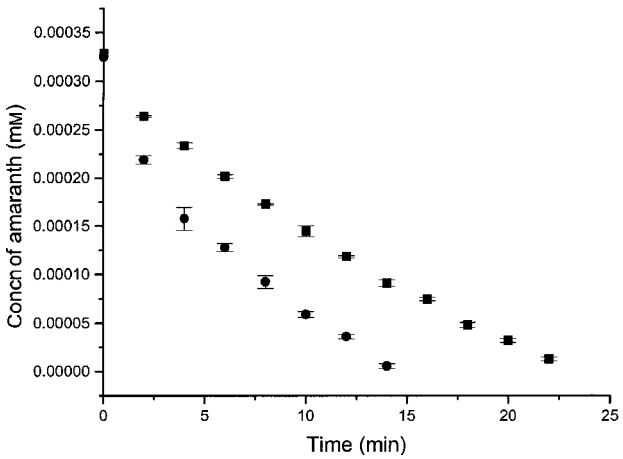

Figure 4 Influence of sonication on the degradation of amaranth: ● = no sonication; ■ = sonication for 20 min at 4°C (n = 3, mean±s.d.).

least part of the azo reductase activity of the anaerobic bacteria of rat caecal content is non-cytoplasmatic and linked with a non-cytosolic centrifugable part of the bacterium. A similar assumption has been made by Kudlich et al (1997), who studied the reduction of azo compounds by *Sphingomonas* sp. BN6 and concluded that azo reductase activity is both cytoplasmatic and membrane bound. In order to keep all the azo reductase activity from the caecal bacteria, it was therefore decided to skip the centrifugation step for all the subsequent in-vitro degradation studies.

The experiments with M2 were carried out in order to investigate the influence of the presence of cofactors on the degradation kinetics (Figure 3). It is clear from this experiment that the degradation speed is increased significantly by the addition of cofactors. Cofactor dependency of the azo reduction rate by anaerobic bacteria has been reported previously by Bragger et al (1997), Semdé et al (1998), Brown (1981) and Chung et al (1978). However, fitting the data to a zero- and a first-order process showed that amaranth degradation can be described adequately by first-order kinetics if additional cofactors are added, while zero-order kinetics were observed in the absence of additional cofactors.

The experiments with M3 show that reducing activity is partially lost when the reaction medium is sonicated (Figure 4). In this case, however, the reaction remained first order. The rationale for sonication is to disrupt the bacterial cells in order to make the intracellular reducing compounds more accessible and thus to increase the azo degradation rate. Dubin & Wright (1975) indeed reported an increased azo reduction rate when bacterial

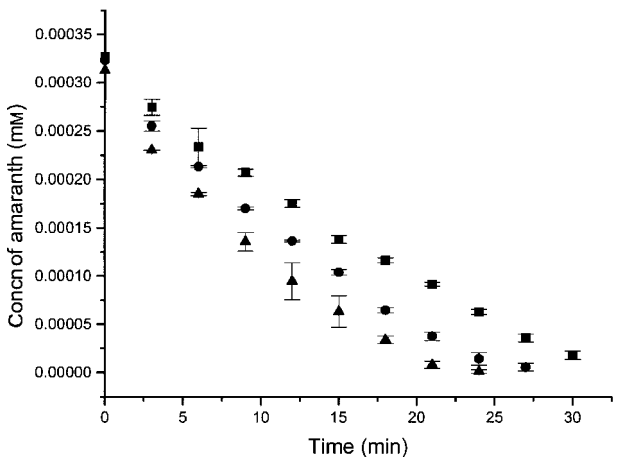

Figure 5 Influence of the concentration of Bz on the degradation of amaranth: ▲ = 0.112 mM; ● = 0.075 mM; ■ = 0.037 mM (n = 3, mean±s.d.).

cell membranes were disrupted. The opposite observations in the present study, namely a decreased azo reduction rate with cell membrane disruption, can probably be explained by the fact that the high amount of energy that is brought into the system by sonication damages part of the reducing enzyme systems, resulting in a decrease in the number of whole cells to effectively mediate the amaranth degradation.

As is significantly illustrated by the experiments using M4, the higher the concentration of the electron shuttle Bz, the higher the degradation rate of the amaranth (Figure 5). These results are in accordance with literature

data (Chung et al 1978; Brown 1981; Bragger et al 1997; Semdé et al 1998), which also show a dependency of the azo reduction rate on the concentration of the electron mediator. In the current experiments, however, a gradual change in the order of the degradation kinetics was observed when the concentration of Bz was decreased. At 0.112 mM of Bz the reaction is first order, while at 0.037 mM the reaction follows zero-order kinetics. At 0.075 mM no significant difference in COD was observed between fitting of the data to a zero- or a first-order process, again indicating that a transitory mechanism rules.

Conclusion

The experiments discussed in this report illustrate that the degradation kinetics of amaranth are highly dependent on the mode of preparation and composition of the degradation medium. In none of the in-vitro experiments described above, however, was the degradation profile of the ex-vivo experiment EV1 observed. This indicates that the selection of an in-vitro azo-reducing system in the evaluation of azo-containing colonic delivery systems should be done with utmost care.

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