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Enhanced anaerobic degradation of polymeric azo compounds by *Escherichia coli* in the presence of low-molecular-weight redox mediators

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Abstract

The effects of the redox mediator lawsone (2-hydroxy-1,4-naphthoquinone) on the ability of *Escherichia coli* to reduce anaerobically polymeric azo compounds were analysed. Two types of polymeric azo compounds were tested, that have been proposed as putative tools for the site-specific targeting of drugs to the colon. The first group of polymers consisted basically of linear chains of polymethacrylic acid or polymethylmethacrylate which were interrupted by subunits of 4,4′-bis-(methacryloylamino)azobenzene. These polymers differed significantly in their hydrophilicity according to the relative proportion of polymethacrylic acid used for the polymerization procedure. The second group of polymers consisted of almost water-insoluble poly(ether-ester)azo polymers that were composed of 4-(6-hydroxyhexyl)oxy-phenylazobenzoate and 16-hydroxyhexadecanoate. The addition of lawsone to the anaerobically incubated cultures of *E. coli* resulted in a pronounced increase in the reduction rates of the water-soluble poly(methacrylate-co-4,4′-bis(methacryloyl-amino)azobenzene) and in a much smaller, but significant, increase in the reduction rates of the hydrophobic poly(ether-ester)azo polymers. An increase in the amount of azo groups resulted, for the hydrophobic poly(ether-ester)azo polymers, in an increased reduction rate in the presence of the redox mediator lawsone.

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Introduction

Azo compounds are widely used for the dyeing of food, paper, leather, textiles and other articles. Most azo dyes are not degraded under aerobic conditions in conventional sewage treatment plants, but are extensively reduced by microorganisms under anaerobic conditions (Chung et al 1992; Stolz 2001). Our laboratory has studied, during recent years, the anaerobic microbial metabolism of sulfonated azo dyes, that are widely used as dyestuffs in the textile and food industry. In these studies, it was observed that the unspecific anaerobic reduction of sulfonated azo dyes may be catalysed by redox mediators, such as lawsone (2-hydroxy-1,4-naphthoquinone) or anthraquinone-2-sulfonate, which enable the bacterial cells under anaerobic conditions to transfer redox equivalents to the azo dyes. This reaction is extremely unspecific because only the reduction of the quinones to the corresponding hydroquinones is an enzymatic process, while the reductive cleavage of the azo bond by the hydroquinones is a purely chemical reduction which can occur extracellularly and therefore does not require transport of the highly polar sulfonated azo compounds into the cells (Kudlich et al 1997; Russ et al 2000; Stolz 2001).

Azo compounds are not only used for dyeing procedures, but they are also used as oral colon-specific drug delivery systems. This application of azo compounds is based on the observation that, under the anaerobic conditions of the large intestine, various azo compounds are reduced by the intestinal flora of the colon (Ryan et al 1968; Chung et al 1992), while they are stable during passage through the stomach and small intestine. This effect has been exploited for almost 30 years for the reductive release of 5-aminosalicylate from the azo compound sulphasalazine (salicylazosulphapyridine) in the colon for the treatment of the inflammatory bowel diseases ulcerative colitis and

Crohn's disease (Peppercorn & Goldman 1972; Van Hees et al 1979). During recent years several attempts have been made to synthesize and utilize polymeric azo compounds for the selective release of drugs into the colon. This may be achieved by utilization of azo groups in prodrugs, biodegradable hydrogels or coatings, which release under the appropriate conditions the pharmaceutical active ingredients into the colon (Kopecek et al 1992; Lloyd et al 1994; Shanta et al 1995; Ghandehari et al 1997; Van den Mooter et al 1997; Kakoulides et al 1998; Kinget et al 1998).

The reductive cleavage of polymeric azo compounds by microorganisms requires extracellular reactions, because it is generally assumed that large polymeric molecules are not transported through the cell membranes. This is especially relevant for bacterial cells that generally do not show endocystosis (Madigan et al 2000). This situation resembles that observed for the sulfonated azo dyes, which are, because of their high polarity, also presumed not to pass the cell membranes (Mechsner & Wuhrmann 1982). We recently demonstrated that the addition of redox mediators, lawsone or anthraquinone-2-sulfonate, to cultures of *Escherichia coli* (and various other bacterial species) resulted in a significant increase in the reduction rates for sulfonated azo dyes (Kudlich et al 1997; Rau et al 2002). We assumed that this could also allow the reduction of polymeric azo compounds which are excluded by their molecular weight from an intracellular reduction. We therefore tested the effects of the redox mediator lawsone on the anaerobic reduction of different types of hydrophilic and hydrophobic azo polymers by *E. coli*.

Materials and Methods

Chemicals

For the synthesis of poly[methacrylic acid-co-4,4′-bis-(methacryloylamino)azobenzene], polyacrylic acid and 4,4′-bis-(methacryloylamino)azobenzene (BMAAB) were dissolved in distilled methanol and 2,2′-azobisisobutyronitrile (1% w/w towards monomers) was added. The solution was purged with nitrogen for 10 min and then placed at 60°C for 24 h. Finally, the polymer was isolated by repetitive precipitation in diethyl ether. The synthesis of the poly(ether-ester)azo polymers has been described previously (Van den Mooter 1994; Samyn et al 1995). Lawsone was supplied by Aldrich (Steinheim, Germany). The sources of all other chemicals have been described before (Kudlich et al 1997; Russ et al 2000; Rau et al 2002).

Cultivation of bacteria

Escherichia coli JM 109 was used in most experiments. The strain was grown in Nutrient Broth to the late exponential growth phase. The cells were harvested by centrifugation, washed with Na/K phosphate buffer (50 mM, pH 7.7) and finally re-suspended in Na/K phosphate buffer to an optical density ($OD_{546\ nm}$) of approximately 5.

Determination of protein content

The protein content of whole cells and cell membranes were determined using the Biuret method and bovine serum albumin as standard (Schmidt et al 1963).

Conversion of the hydrophilic water-soluble azo compounds

Of all the substrates tested in this study, only the monomer BMAAB and the polymer poly[methacrylic acid-co-4,4′-bis-(methacryloylamino)azobenzene] with 1% azo compound showed sufficient solubility in water. This allowed a spectrophotometric assay in which the reduction of the azo bond was directly measured in aqueous solution. The conversion of these substrates was assayed under anaerobic conditions in rubber-stoppered glass cuvettes (path length d = 1 cm). The reaction mixtures contained in 1 mL: 50 μmol Na/K-phosphate buffer (pH 7.7), 10 μmol glucose, whole cells of *E. coli* (corresponding to an optical density $OD_{546\ nm}$ = 0.5) and 69 or 57 nmol azo bonds for BMAAB or poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene], respectively. The molar extinction coefficients for the azo groups of BMAAB and the poly[methacrylic acid-co-4,4′-bis-(methacryloylamino)azobenzene] at λ = 364 nm were experimentally determined as $\epsilon_{364BMAAB}$ = 26.5 and ϵ_{a364} = 19.7 $mM^{-1}\ cm^{-1}$, respectively. The spectrophotometric assays were performed at 364 nm because this is an isosbestic point for the interconversion of lawsone (LQ) and its corresponding hydroquinone (ϵ_{364LQ} = 1.0). Thus it was possible to determine the decrease in the concentration of the azo compound spectrophotometrically without interference from the redox reactions of lawsone. The concentration of lawsone was determined simultaneously at λ = 450 nm (ϵ_{450LQ} = 2.8 $mM^{-1}\ cm^{-1}$) and corrected for the absorption caused by the respective azo compound ($\epsilon_{450BMAAB}$ = 33 $mM^{-1}\ cm^{-1}$; $\epsilon_{\alpha 450}$ = 2.7 $mM^{-1}\ cm^{-1}$). The UV/Vis spectrum of the reduced form of lawsone shows no significant extinction at this wavelength. The calculated concentrations of lawsone were corrected for the changes in absorbance at 450 nm caused by the turnover of the azo compounds. For the determination of the reaction rates, the absorbances at 364 nm and 450 nm were also corrected for the absorbance caused by the bacterial cells at the respective wavelength using a separate cuvette with *E. coli* cells suspended in the same buffer as blank.

The concentrations, c, of BMAAB, lawsone (LQ) and reduced lawsone (LQ_{red} = 1,2,4-trihydroxynaphthalene) were simultaneously determined in one cuvette according to the following formulas (E = extinction at nm, $c_{LQ\ Start}$ = initial concentration of lawsone):

$$c_{BMAAB} = \{E_{364\ nm} - (c_{LQ\ Start} \cdot d \cdot \epsilon_{364LQ})\} \cdot \{d \cdot \epsilon_{364BMAAB}\}^{-1} \quad (1)$$

$$c_{LQ} = \{E_{450\ nm} - (c_{BMAAB} \cdot d \cdot \epsilon_{450BMAAB})\} \cdot \{d \cdot \epsilon_{450LQ}\}^{-1} \quad (2)$$

$$c_{LQred} = c_{LQ\ Start} - c_{LQ} \quad (3)$$

Similarly, for the turnover of poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] in the presence of lawsone and *E. coli*, the following equations were used (c_α = concentration of azo groups in the polymer):

$$c_\alpha = \{E_{364\ nm} - (c_{LQ\ Start} \cdot d \cdot \epsilon_{364LQ})\} \cdot \{d \cdot \epsilon_{\alpha 364}\}^{-1} \quad (4)$$

$$c_{LQ} = \{E_{450\ nm} - (c_\alpha \cdot d \cdot \epsilon_{\alpha 450})\} \cdot \{d \cdot \epsilon_{450LQ}\}^{-1} \quad (5)$$

$$c_{LQred} = c_{LQ\ Start} - c_{LQ} \quad (6)$$

Preparation of films of the poly(ether-ester)azo polymers

The polymers were dissolved in a solvent that contained about 0.5 mL of 1,1,1,3,3,3-hexafluoroisopropanol in 10 mL of chloroform to a final concentration of 3.5 g L^{-1}. Finally, 50 μL of this solution of the azo polymer was put on a Teflon membrane (about 3 × 1 cm) and the solvent was evaporated with a stream of nitrogen, leaving a yellow spot of the polymer on the teflon membrane. The membrane with the azo polymer was tightened with a piece of firm plastic, about the same size as the membrane, and charged with two cramps at both ends to prevent clumping of the membrane during subsequent incubation.

Turnover experiments with the water-insoluble azo polymers

The experiments were performed in rubber-stoppered serum bottles (25 mL) containing, in 10 mL : 100 mM Na/K phosphate buffer (pH 7.7), 10 mM glucose, cells of *E. coli* with an optical density ($OD_{546\ nm}$) of approximately 0.5 and the redox mediators. The devices with the azo polymer attached to the Teflon membrane were transferred to the filled serum bottles and oxygen was removed from the medium by repeated evacuation and flushing with nitrogen gas. The serum bottles were incubated in the dark at 37°C on a rotary shaker (140 rev min^{-1}). After different time intervals, the membranes with the azo polymers were removed, rinsed with water and dried by gently passing a stream of nitrogen over the membranes. The azo polymer was dissolved from the dry membranes by the addition of 1 mL of chloroform and gentle stirring for about 20 s. The concentration of the azo polymer dissolved in chloroform was determined by measuring the absorbance at 356 nm. A control experiment in the absence of bacteria was always treated in the same way to exclude any spontaneous abiological loss of the azo polymers from the membranes.

Quantitation of the content of azo groups in the water-insoluble polymers

Stock solutions (3–16 mg mL^{-1}) of the water-insoluble polymers were prepared in a mixture of chloroform and 1,1,1,3,3,3-hexafluoroisopropanol (99 : 1, v/v). These solutions demonstrated an absorbance maximum (λ_{max}) at 356 nm, which was due to the presence of the azo group in the polymers and thus allowed a quantitation of the amounts of azo groups present in the polymers. The stock solutions were diluted in the same solvent system and calibration curves determined in the dark. The amount of the azo groups present in the polymers was then calculated from the known proportion of the azo-group containing monomers used for the synthesis of the polymers.

Statistical analysis

The experiments were routinely performed in triplicate and means ± s.d. for each experiment were calculated. The means of individual treatments were compared using a Mann–Whitney U test, $P > 0.05$ denoting significance.

Results

Conversion of hydrophilic methacrylic-acid-derived azo polymers

Two groups of polymeric azo compounds were investigated. The first group consisted of linear chains of polymethacrylic acid or polymethylmethacrylate (for the structures of the monomers used for synthesis, see Figure 1) which were interrupted by subunits of 4,4′-bis(methacryloylamino)azobenzene (azo compound content about 1%). These polymers differed significantly in their water solubility. The polymers synthesized from polymethacrylic acid and 4,4′-bis(methacryloylamino)-azobenzene (BMAAB) were water soluble in the concentration range analysed. In contrast, the polymethyl-

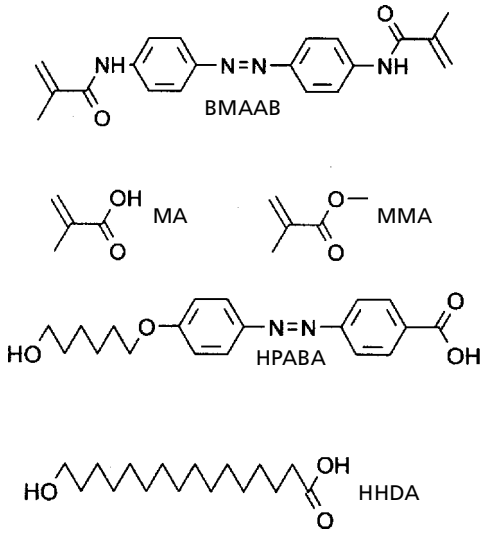

Figure 1 Chemical structures of the monomers used for the synthesis of the methacrylic-acid-derived azo polymers and the poly(ether-ester)azo polymers. BMAAB, 4,4′-bis(methacryloylamino)azobenzene; MA, methacrylate; MMA, methylmethacrylate; HPABA, 4-(6-hydroxyhexyl)oxy-phenylazobenzoate; HHDA, 16-hydroxyhexadecanoate.

Table 1 Characteristics of the azo compounds analysed, reaction conditions used and specific activities observed for the reduction of the azo compounds.

Entry	Compound	Substance	Composition[a]	α[b] (mmol g^{-1})	Solvent used	λ max (nm)	ε_α(l $mmol^{-1}$ cm^{-1})	Test system	Spec. activity (U_α g^{-1})	
									+0.25 mM lawsone	Without lawsone
1	Poly[methacrylate-co-4,4′-bis (methacryloylamino) azobenzene]	A	1% BMAAB 99% MA	0.029	H_2O	364	19.7	Dissolved in aqueous phase	1.2±0.1	< 0.01±0.01
2	4,4′-Bis(methacryloylamino) azobenzene	—	100% BMAAB	2.874	H_2O	389/427	37.6/36.1	Dissolved in aqueous phase	1.9±0.2	0.09±0.01
3	Poly[methylmethacrylate-co-4, 4′-bis(methacryloylamino) azobenzene]	B	1% BMAAB 99% MMA	0.029	$CHCl_3$	356	29.3	Water-insoluble film	< 0.0001±0.0001	< 0.0001±0.0001
4	Poly[(4-(4-(6-hydroxyhexyl) oxy)phenylazo) benzoate-co-16-hydroxyhexadecananoate]	C	5% HPABA 95% HHDA	0.147	$CHCl_3$	356	34	Water-insoluble film	0.0006±0.0001	< 0.0001±0.0001
5	as C	D	40% HPABA 60% HHDA	1.176	$CHCl_3$	356	26.7	Water-insoluble film	0.0014±0.0001	0.0005±0.0001

[a]The abbreviations refer to Figure 1. [b]The α-values given refer to the concentration of the azo bond in mmol per gram of the respective azo compound.

methacrylate derivatives showed only a significant solubility in organic solvents (Table 1).

The microbial reduction of the water-soluble poly-[methacrylic acid-co-4,4′-bis(methacryloylamino)azo-benzene] (Table 1, polymer A) was assayed in a purely aqueous system in a spectrophotometric assay (Figure 2A). The polymer demonstrated an absorbance maximum (λ_{max}) at 364 nm. The assay contained, under anaerobic conditions, the polymer (2.0 mg mL^{-1}), cells of *E. coli* ($OD_{546\ nm}$ = 0.5) and glucose (10 mM) in the absence or presence of lawsone (0.25 mM). Every 10 min, the changes in the absorbance were analysed at 364 nm and 450 nm (absorption maximum of lawsone). After correction of the values determined for the absorbance caused by the cells within the cuvette at the relevant wavelength, it was shown that the cells reduced lawsone almost completely within 30 min and that when sufficient amounts of reduced lawsone were present, a slow reduction of the azo bonds started (Figure 2A). In contrast, almost no turnover of the polymer by the cells was observed in the absence of lawsone (Figure 2A).

The results clearly demonstrated that the addition of the redox mediator lawsone significantly increased the turnover of the polymeric azo compound. Therefore, to analyse the effects of the polymeric structure on the ability of redox mediators to enhance the anaerobic conversion of azo compounds in more detail, the turnover of the azo compound BMAAB, which represents the monomeric structural unit containing the azo bond in poly(methacrylic acid)-co-4,4′-bis(methacryloylamino)azo-benzene (Figure 1; Table 1, entry 2), was studied. For the monomeric BMAAB, basically the same direct spectrophotometric test system as described above for poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] was used. When cells of *E. coli* were incubated in the presence of lawsone with BMAAB (Figure 2B), it was also observed that lawsone was rapidly reduced by the cells but, surprisingly, the following reduction of the azo bond was only slightly enhanced compared with the reaction observed with poly[methacrylic acid-co-4,4′-bis(methacryloylamino)-azobenzene] (Figure 2A, B; Table 1).

Influence of the water solubility on the redox-mediator-dependent reduction of methacrylic-acid-derived azo polymers

The results obtained with poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] suggested that the reduced form of lawsone was able to reduce the azo bonds within polymeric structures. Poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] (polymer A) showed a significant water solubility, which might facilitate a reaction with the reduced form of lawsone. Many of the presumed applications of azo polymers involve water-insoluble azo compounds (e.g. for the coating of pharmaceuticals). Therefore, a hydrophobic derivative of poly[methacrylic acid-co-4,4′-bis(methacryloylamino)-azobenzene] was investigated, in which the acrylic-acid residues were esterified by methyl groups (Figure 1; Table 1, entry 3, polymer B). This azo polymer was almost insoluble in water and was therefore dissolved in a chloroform solvent. These solutions were spread on teflon membranes, the chloroform evaporated and the remaining film of the polymer on the teflon membrane was used for the biodegradation studies. In a typical experiment, the membranes (with 0.8 mg of azo polymer) were incubated under anaerobic conditions in serum bottles in 10 mL Na/K-phosphate buffer solution with cells of *E. coli* (optical density $OD_{546\ nm}$ of about 0.5) in the presence of glucose (10 mM) and lawsone (0.25 mM). The teflon membranes with the attached azo polymers were removed after different time intervals, the azo compound dissolved in a chloroform solvent and the concentration of the azo compounds determined spectrophotometrically at 356 nm, which was the absorbance maximum of the polymer. No significant

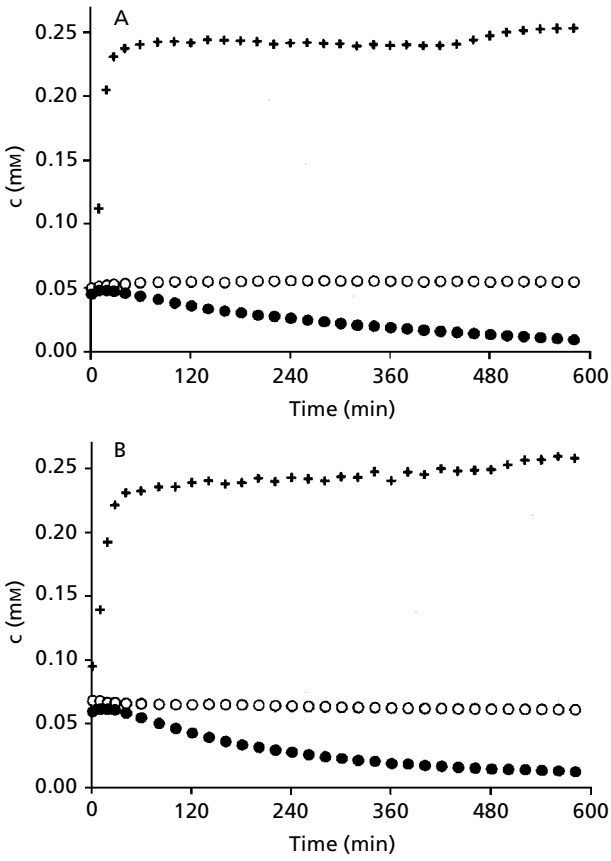

Figure 2 Influence of lawsone on the anaerobic reduction of water-soluble methacrylic-acid-derived azo polymers. The reaction mixtures contained, in 1-mL cuvettes under anaerobic conditions: 50 mM Na/K-phosphate buffer (pH 7.7), cells of *E. coli* ($OD_{546\,nm}$ = 0.5), 10 mM glucose and either poly[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] (Table 1, polymer A) corresponding to 57 μM of azo groups (A) or BMAAB (69 μM; B). The turnover of the azo groups (●, ○) was determined in the presence (filled symbols) or absence (open symbols) of lawsone (0.25 mM). Simultaneously, the concentration of the reduced form of lawsone (+) was measured in both assay systems. The concentrations of the respective compounds were determined spectrophotometrically by the continuous simultaneous analysis of the reaction mixtures at λ = 364 nm and 450 nm and corrected for the cell sedimentation which was determined in a separate cuvette (see Materials and Methods section).

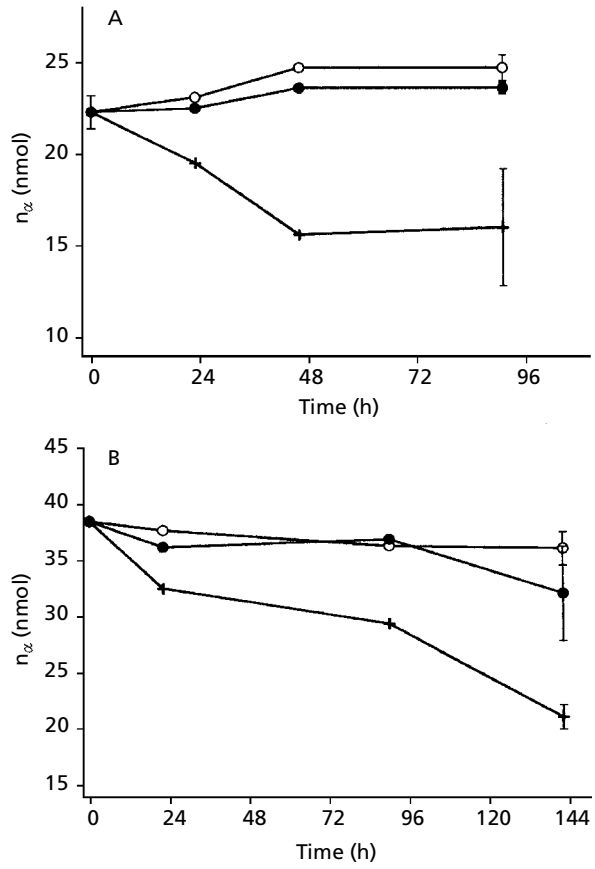

Figure 3 Degradation of films of poly(ether-ester)azo polymers by *E. coli* in the presence or absence of the redox mediator lawsone. The films of the poly(ether-ester)azo polymers (polymer C with 5% azo groups (A) or polymer D with 40% azo groups (B)) were prepared on teflon membranes as described in the Materials and Methods section. The teflon membranes with the polymer film were incubated anaerobically with cells of *E. coli* JM109 ($OD_{546\,nm}$ = 0.5–0.7) in the presence of glucose (10 mM). The serum bottles were incubated in the dark at 37°C on a rotary shaker and the membranes were removed after different time intervals and rinsed with water. The azo polymer was dissolved in chloroform and the amounts of the residual azo bonds quantified spectrophotometrically after incubation of the polymer in the presence of cells and lawsone (0.5 mM) (+), with cells in the absence of lawsone (●) or in a cell-free control (○) (see Materials and Methods). The crossbars represent the standard deviation (n = 3). The residual amounts of the azo groups are given as nmol of azo groups totally present on the membranes (n_α).

reduction of the azo groups could be detected in this system in the absence or presence of lawsone within a week.

Poly(ether-ester)azo polymers

The results obtained with the water-soluble poly-[methacrylic acid-co-4,4′-bis(methacryloylamino)azobenzene] (polymer A) and the comparatively water-insoluble ester derivative (polymer B) clearly demonstrated a strong influence of water solubility on the reactivity of the reduced form of lawsone on the azo polymers. The poly(methacrylate) derivatives analysed contained only about 1% of the azo monomer (Table 1). This low amount of azo groups within the polymers might result in a low degree of availability of the azo groups for the reduced form of lawsone within the polymers. Furthermore, the relatively low amount of azo groups reduced the sensitivity of the spectrophotometric degradation tests. Therefore, a second group of almost-water-insoluble polymers was investigated, consisting of poly(ether-ester)azo polymers which were composed of 4-(6-hydroxyhexyl)oxyphenylazobenzoate and 16-hydroxyhexadecanoate (Figure 1). The two polymers contained significantly higher amounts of azo

groups (5% or 40%) than the poly(methacrylate) polymers and differed from each other by the relative proportion of 16-hydroxydecanoic acid (95% or 60%) that was copolymerized into the azo polymers (Table 1, polymers C and D). For the polymer with a content of about 40% azo monomers, an average molecular weight of 2×10^4 has been calculated previously (Van den Mooter 1994). These azo polymers were almost insoluble in water and were therefore spread on teflon membranes and analysed in the same assay as described above for the poly[ethylmethacrylate-co-4,4′-bis(methacryloylamino)-azobenzene]. Thus, a significant reduction in the concentrations of the azo group was observed after incubation of the poly(ether-ester)azo polymers with the cells and the redox mediator lawsone (Figure 3). In contrast, no decrease in the concentration of the azo bonds was found in the absence of the cells or the redox mediators (Figure 3). It was observed, reproducibly, that only about 30–50% of the azo groups could be converted by the bacteria in the presence of the redox mediator. This may be explained by diffusion limitations preventing the action of the reduced redox mediator on azo groups that are located in deeper areas of the film.

Is there a true cleavage of the azo groups in the polymeric azo compounds?

The results presented thus far clearly demonstrated that the addition of lawsone resulted in a significant increased reduction of the azo bonds in water-soluble and also water-insoluble polymeric azo compounds. The spectrophotometric assays used during this study could not distinguish between a two-electron-requiring reduction of the azo groups to the corresponding hydrazines (which would leave the backbone of the polymers intact) and a true cleavage of the azo polymers to the amines (which would result in a true destruction of the polymers) (Schacht et al 1996). The behaviour of the water-insoluble polymers was tested with the poly[(4-(4-(6-hydroxyhexyl)oxy)phenylazo)benzoate-co-16-hydroxyhexadecananoate] with 40% azo-content (polymer D) that had shown the highest reaction rate out of all the water-insoluble polymers tested. Several films of the azo polymer were incubated anaerobically with cells of *E. coli* and lawsone, as described above. After 6 days of incubation, the films were removed from the serum bottles and a significant decrease in the amount of azo groups was determined after re-dissolution of the films in chloroform (Figure 4, entries A and B vs entry E). A further set of films, incubated anaerobically with cells of *E. coli*, glucose and lawsone (identical treatment as the film shown in Figure 4, entry E), were dried and stored under air (Figure 4, entries F–I) or under a nitrogen atmosphere for different time intervals up to 52 days. Thus it was found that the decolourization of the films that was observed under anaerobic conditions was almost completely reversed after several weeks of storage of the initially decolourized films under air. This suggested that the azo groups of poly[(4-(4-(6-hydroxyhexyl)oxy)phenylazo)benzoate-co-16-hydroxyhexadecananoate] (polymer D) were reduced in the presence of lawsone mainly to hydrazo groups, that were slowly re-oxidized to azo groups in the presence of molecular oxygen.

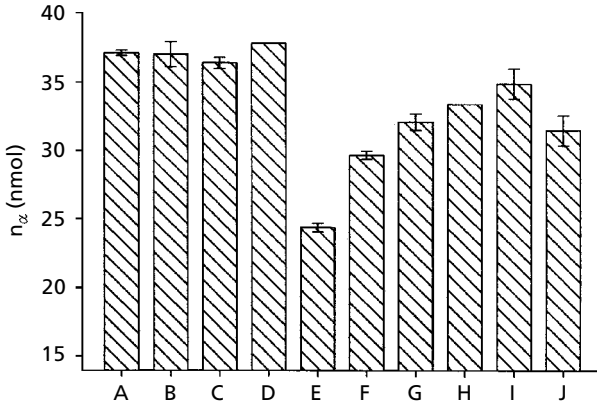

Figure 4 Re-oxidation of anaerobically incubated decolourized films of a poly(ether-ester)azo polymer after re-introduction of oxygen. The individual serum bottles (B–J) contained, under anaerobic conditions: 37 μg of the poly(ether-ester)azo polymers (40% azo content, polymer D in Table 1), glucose (10 mM), cells of *E. coli* (OD_{546n} = 0.5) and lawsone (0.5 mM). Control experiments were devoid of cells and lawsone (B), lawsone (C) or cells (D). In a further control (A) three independent films of the polymer were analysed for the content of azo groups without an anaerobic incubation. The serum bottles B–J were shaken at 37°C for 6 days and the remaining polymer films removed and dried under nitrogen. The amount of azo groups present in the films was directly analysed after dissolving the films in the chloroform solvent in serum bottles B, C, D and E. The remaining dried films were stored under air (F, G, H and I) or under a nitrogen atmosphere (J) for 1 (F), 4 (G), 8 (H) or 52 (I, J) days before the films were dissolved in chloroform and the amount of azo groups determined spectrophotometrically as described above.

Discussion

This study clearly demonstrated that the redox mediator lawsone increased the ability of *E. coli* to reduce different types of hydrophilic and hydrophobic polymeric azo compounds, which carry the azo group in the backbone of the polymer. Because it was previously shown that various bacterial species are able to reduce lawsone (and other quinoide redox mediators) (Kudlich et al 1997; Field et al 2000; Rau et al 2002), this indicated that lawsone and other redox mediators may be important for the metabolism of azo compounds which are restricted in their permeability through microbial membranes because of their charge or size. A positive effect of the putative redox mediator benzylviologen has already been described for some polymeric azo compounds carrying the azo groups connected via side-chains to the backbone of the polymers. Thus, Brown (1981) demonstrated, for water-soluble linear poly(vinyl amine) derivatives carrying the azo dyes sunset yellow and tartrazine coupled via sulfonamide linkages to the polymer backbone, that resting cells of a wide variety of

anaerobic bacteria and also a suspension of rat caecal content could reduce the polymeric azo compounds in the presence of benzylviologen or FMN with a 2- to 12-fold increased reduction rate. Similarly, it was observed for water-soluble *N*-(2-hydroxypropyl)methacrylamide copolymers containing 5-aminosalicylate using cell-free extracts from rat caecum that the addition of benzyl viologen resulted in an increase in the rate of azo reduction (Kopeckova & Kopecek 1990; Ghandehari et al 1997). In those studies, almost exclusively benzylviologen or flavine nucleotides were used as mediator compounds and in many of these studies the effects of mediators on the azoreductase activity were determined with cell-free extracts. In this study, it was demonstrated that quinones fulfil mediator functions transferring reduction equivalents from whole cells to the azo compounds. Quinones are, in contrast to benzylviologen, almost ubiquitous in the environment and are also important constituents of edible plants. Furthermore, certain quinoide redox mediators (e.g. 2-amino-3-carboxy-1,4-naphthoquinone) are even formed by gastrointestinal bacteria such as *Propionibacterium freudenreichii* (Yamazaki et al 1998, 1999). Therefore, it appears that also in the colon, quinones are much more probable redox mediators than are viologens or flavin nucleotides which should not accumulate in significant amounts extracellularly in biological systems.

The results of this study, together with those of some previous studies using different polymeric compounds, may allow some generalizations about the factors influencing the reduction of polymeric azo compounds. Firstly, it appears, from the comparison of the reaction rates observed for the monomeric azo compound BMAAB and the hydrophilic copolymerisate of BMAAB and methacrylic acid, that the polymer was only slightly reduced more slowly than the monomer. This result suggests that the reaction of the mediators is almost independent of the size of the azo compound. The apparent independence of the molecular weight of the azo compounds and the reduction rates has also previously been observed for water-soluble polyethylene derivatives carrying sunset yellow or tartrazine attached to side-chains or *N*-(2-hydroxypropyl)methacrylamide copolymers containing 5-aminosalicylate using in-vivo experiments with rats or cell-free extracts from rat caecum (Honohan et al 1977; Kopeckova & Kopecek 1990). These results suggest that in water-soluble polymers which carry the azo groups either attached to side-chains as described in the literature or in the main chain as described in this study, the azo group is sufficiently accessible for the low-molecular redox mediators and that therefore a polymeric structure does not prevent the reduction of the azo group.

The comparison of the reduction rates observed with the monomeric BMAAB, the somewhat better water-soluble poly[methacrylate-co-4,4′-bis(methacryloylamino)azobenzene] (polymer A) and the hydrophobic poly[methylmethacrylate-co-4,4′-bis(methacryloylamino)azobenzene] (polymer B) suggested that the water solubility of the substrates had a stronger influence on the ability of the redox mediators to reduce the substrates than the pure size of the azo compounds. Thus it was observed in this study that the water-soluble poly[methacrylic acid-co-4,4′-bis-(methacryloylamino)azobenzene] was converted under our test conditions in the presence of lawsone with more than 1000-fold higher rates compared with the almost water-insoluble poly(ether-ester)azo polymers or the hydrophobic poly[methylmethacrylate-co-4,4′-bis(methacryloylamino)azobenzene] (polymer B) (Table 1). These results basically coincide with studies about the fate of polymeric azo compounds in the colon which suggest that the reduction of different azo polymers by the bacterial flora of the intestine may be mainly influenced by the hydrophilicity of the azo polymers and that only polymers with a sufficient degree of hydrophilicity can be degraded within an acceptable period of time (Van den Mooter et al 1993; Kopeckova et al 1994).

In the *E. coli*/lawsone system it was found, for the hydrophobic poly(ether-ester)azo polymers, that the highest degree of degradation was observed with the polymer containing the highest amount of azo groups. A similar observation was made earlier by Samyn et al (1995) when these polymers were incubated with cell extracts prepared from the caecal content of rats.

In contrast to the poly(ether-ester)azo polymers (polymers C and D), no reduction of the azo group was observed with the other hydrophobic polymer (polymer C; poly[methylmethacrylate-co-4,4′-bis(methacryloylamino)-azobenzene]). This effect could be possibly explained by the differences in the amounts of the azo compounds present in the polymers (5–40% for the poly(ether-ester)azo polymers vs only 1% for the poly[methylmethacrylate-co-4,4′-bis-(methacryloylamino)azobenzene]). The amount of polymer used was standardized according to the concentration of azo groups in the tests because of the sensitivity of the final spectrophotometric assay for the azo groups. This resulted, under the standard reaction conditions, in a much higher content of polymeric material during the assays with polymers containing only low amounts of azo groups. Thus the low degree of degradability of poly[methylmethacrylate-co-4,4′-bis(methacryloylamino)azob enzene] compared with the poly(ether-ester)azo polymers could be due to the different structure of the monomers containing the azo group or to a reduced availability of the fewer azo groups within the poly[methylmethacrylate-co-4,4′-bis-(methacryloylamino)azobenzene] for an attack of the redox mediators.

The results obtained with the poly(ether-ester)azo polymers suggested that these water-insoluble azo compounds were reduced only to the hydrazine stage in the *E. coli*/lawsone system. The reduction of hydrophobic azo polymers to the corresponding hydrazines has also been reported for hydrophobic polyamides containing azo groups in the backbone in the polymer using a simulated human intestinal microbial ecosystem (Schacht et al 1996) and for a linear-type azo-containing polyurethane with an anaerobic bacterial culture prepared from human faeces (Kimura et al 1992; Ueda et al 1996; Yamaoka et al 2000). Although decolourization was observed in the *E. coli*/lawsone system with the hydrophilic polymethacrylate (polymer A), we were also unable to definitively prove a true fission of the azo bonds for this type of polymer

by viscosity measurements (data not shown). A putative strategy to obtain a complete reduction of azo polymers to the amines might be the introduction of a hydroxy group in the *ortho* position to the azo group as it is found in almost all commercially important azo dyes. This may allow a complete reduction of the azo bonds, because it was previously repeatedly suggested for simple azo dyes, that the reduction of the azo groups would, in principle, only result in the formation of the hydrazine, which in the case of an *ortho*- or *para*-hydroxy (or amino-) azobenzene would then undergo disproportionation to an aminobenzene and an *ortho*- or *para*-iminoquinone, which then would finally be reduced to the aminohydroxybenzene (Florence 1965; Dubin & Wright 1975). This kind of disproportionation cannot take place with the azo polymers previously synthesized, which do not carry hydroxy substituents on the aromatic ring in the neighbouring position to the azo-group.

In conclusion, the results obtained using the well-defined system *E. coli* plus lawsone clearly resembled the results obtained in previous studies using different types of polymeric azo compounds and much more complex biological systems. This suggests that in the complex system of the colon, presumably also quinoide mediator compounds are important for the reduction of polymeric azo compounds. The results also suggest that the metabolic fate of polymeric azo compounds can be assayed initially by an anaerobic incubation with *E. coli* in the presence of a quinoide mediator compound.

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