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Investigations into the azo reducing activity of a common colonic microorganism

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Abstract

This work was undertaken to study some factors affecting the bacterial reduction (cleavage) of azo compounds, knowledge of which will be of use in the development of azo cross-linked polymers for colon-specific drug delivery. A common colonic bacterium, *Bacteroides fragilis* was used as test organism and the reduction of azo dyes amaranth, Orange II and tartrazine were studied; also a model azo compound, 4,4′-dihydroxyazobenzene. It was found that the azo compounds were reduced at different rates and the rate of reduction could be correlated with the half-wave (redox) potential of the azo compounds. 4,4′-Dihydroxyazobenzene ($E_{1/2}$ −470 mV) was reduced at the fastest rate of 0.75 mol l^{-1} h^{-1}, amaranth ($E_{1/2}$ −568 mV) at 0.30 mol l^{-1} h^{-1}, Orange II ($E_{1/2}$ −648 mV) at 0.2 mol l^{-1} h^{-1} and tartrazine ($E_{1/2}$ −700 mV) at 0.08 mol l^{-1} h^{-1}. Similar observations were made with another colonic bacterium *Eubacterium limosum*. Reduction of 4,4′-dihydroxyazobenzene did not occur under conditions of aeration, but was enhanced by the low molecular weight electron carrier benzyl viologen, with time for 50% azo reduction being decreased from 120 min to 30 min. These studies with a common, numerically important, colonic bacterium indicate that the reduction of an azopolymer may be influenced by the chemical nature of the azo compound used as cross-linker. © 1997 Elsevier Science B.V.

Keywords: Azo reduction; Azo dyes; Colonic drug delivery; *Bacteroides fragilis*; Redox potential

1. Introduction

The in vivo metabolism of azo compounds has been acknowledged for many years. The earliest recorded studies were those of Sisley and Porscher (1911), who identified sulphonic acid in the urine of dogs fed the azo dye Orange I. Radomski and Mellinger (1962) demonstrated that important modifications of azo dyes took place in the gut of experimental animals before absorption, and Pep-

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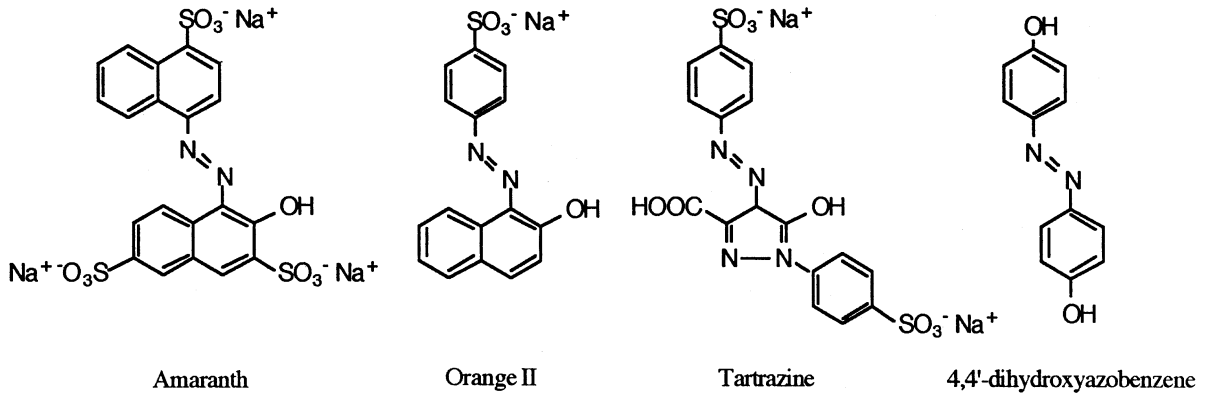

Fig. 1. Structures of the azo compounds studied.

percorn and Goldman (1972) showed that sulphasalazine is split by the gut flora into the active constituent 5-aminosalicylic acid and the carrier molecule sulphapyridine.

Since then, a large number of papers have been published on the subject of bacterial azo reduction, many pertaining to the mutagenic/carcinogenic activation of azo dyes (Chung, 1982; Cerniglia et al., 1986), and to the utilisation of azo reductase in drug delivery systems (Saffran et al., 1986; Bronsted and Kopecek, 1992; Van den Mooter et al., 1993, 1994, 1995; Schacht et al., 1996).

The initial work stimulating research into azo polymers for colon-specific drug delivery, performed by Saffran et al. (1986), proposed that microbially reducible azo bonds could be incorporated into a polymeric structure in order to formulate a dosage form coating that would be insoluble in the upper gastrointestinal tract, but susceptible to degradation in the colon. This would then result in breakdown of the coating material and subsequent release of drug.

Much of the current research into development of azo polymers as drug delivery systems is based on the nature of the polymeric backbone. Although Bronsted and Kopecek (1992) and Van den Mooter et al. (1993) have investigated the importance of azo cross-linking agent, the range of compounds investigated is limited. There may be the opportunity to control the rate of reduction of the azo bond by choice of different azo compounds. Recent reviews on azo polymer research have indicated the need for rational design of the azo cross-linker molecules taking into account electronic parameters affecting ease of reduction of the azo bond (Lloyd et al., 1994; Kumar, 1992).

However, the mechanism of, and factors affecting azo reduction are still not fully understood (Chung et al., 1992) and this study was undertaken to examine two aspects of the azo reductase system of relevance to the design of azo cross-linking molecules for colon-specific polymers.

Firstly, the relationship between the redox potential of a range of azo substrates and the ease of reduction of the azo bond was investigated. Previous work by Dubin and Wright (1975) had indicated that this was an important relationship in reduction of dyes by *Proteus vulgaris*; however this organism is not considered to be of great relevance to the human colon (Roxon et al., 1967; Finegold et al., 1974; Borriello et al., 1978). Therefore in the present study, *Bacteroides fragilis* and *Eubacterium limosum* have been used, both of which are strictly anaerobic organisms commonly found as major inhabitants of the human colon (Finegold et al., 1974; Borriello et al., 1978).

Secondly, a model compound, 4,4-dihydroxyazobenzene, was used to investigate some factors affecting the rate of azo reduction, namely sensitivity of azo reduction to the presence of oxygen, acceleration of reduction by redox mediators and increased bacterial cell density, and effect of pH.

The structures of all the azo compounds studied are shown in Fig. 1.

2. Materials

Chemical reagents used were obtained from Sigma unless otherwise stated. Sodium dihydrogen orthophosphate and di-potassium hydrogen orthophosphate were Analar grade from BDH, Poole, UK. 4-Aminophenol, HCl, ammoniacal copper chloride, hydroxylamine hydrochloride, amaranth, Orange II and tartrazine were used as obtained from Aldrich, Gillingham, UK. Materials for the preparation of Wilkens-Chalgren Anaerobe Broth (WCAB), Reinforced Clostridial Medium (RCM), and Agar No. 1 were obtained from Oxoid Ltd., Basingstoke, UK. Cooked Meat Medium (CMM) was obtained from Lab M, Bury, Lancs, UK.

3. Methods—Synthetic and physicochemical

3.1. Synthesis of 4,4′-dihydroxyazobenzene

4,4′-Dihydroxyazobenzene was prepared by modification of the method of Bogoslowski (1946). 4-Aminophenol (10.9 g) was dissolved in 5 M hydrochloric acid (100 ml) and diazotized at 2°C with sodium nitrite (7 g in 20 ml of water). A solution of ammoniacal copper chloride was prepared by treating a solution of copper chloride (19 g) under nitrogen with aqueous ammonia (30 ml, 33% w/w) and hydroxylamine hydrochloride (7 g in 20 ml of water). The diazonium solution was diluted to 5 l with distilled water and added slowly to the stirred ammoniacal copper chloride solution on ice. After 15 min the ice bath was removed and the reaction mixture stirred for a further hour. The product was collected by filtration, washed successively with 30-ml aliquots of ammonia solution (12% w/v) until a clear filtrate was obtained. The solid was then washed with water (2×50 ml), 2 M hydrochloric acid (2×50 ml) and water (2×50 ml). The product was recrystallised from ethanol and dried in vacuo. Yield 19.2%, m.p.: 214–215°C. The infra-red and ^{1}H NMR spectroscopy were consistent with the proposed structure. The CHN analysis indicated that the isolated product was the monohydrate. Analysis (calculated value): Carbon 62.0% (62.1%), Hydrogen 4.8% (5.2%), Nitrogen 12.0% (12.1%).

3.2. Half-wave potential measurement of azo substrates

The half-wave potentials of the water soluble azo dyes amaranth, Orange II and tartrazine and 4,4′-dihydroxyazobenzene were determined as follows: solutions of the azo compounds (0.1 mM) were prepared in 0.02 M phosphate buffer (pH 7) and DC current-voltage curves were obtained at 21 ± 1°C, by means of a recording polarograph (Polarographic Detector Model 303, Princeton Applied Research, NJ, USA) using a polarizing range of -200 to -950 mV versus Ag/AgCl reference electrode, a current range of 0.2 mA and a 5 mV s^{-1} sweep.

4. Methods—Bacteriological techniques

4.1. Preparation of pre-reduced, anaerobically sterile (PRAS) media

The guidelines of Holdeman and Moore (1973) were adopted for the preparation of PRAS media.

4.2. Preparation of Bacteroides fragilis test suspension

Freeze-dried *B. fragilis* was reconstituted into 20 ml sterile CMM and incubated for 3 days at 37°C to produce a pure culture. This was then streaked onto WCAB agar slopes in 20-ml Universal bottles. After a further 3 days of incubation, these stock slopes were stored for up to 6 months at 4°C until used to inoculate fresh CMM.

To produce a test suspension of *B. fragilis*, 1 ml of the CMM bacterial culture was removed aseptically and used to inoculate 100 ml of WCAB

(PRAS) medium. Incubation at 37°C for 14 h yielded a stationary phase culture containing 1 mg/ml (dry wt.) of bacterial cells. Stock cultures and WCAB cultures used in experiments were checked regularly for contamination by streaking onto agar, incubating under both anaerobic and aerobic conditions and then Gram staining resultant colonies.

Anaerobic conditions were generated by use of an anaerobic jar and Gaspak ($CO_2/N_2/H_2$, 80:15:5) sachets (Oxoid Ltd., Basingstoke, UK).

4.3. Enumeration of bacteria

The density of bacterial cells was calculated by a method adapted from Brown (1981). Aliquots of the test suspension (20 ml) were accurately measured and placed in pre-weighed centrifuge tubes. The samples were centrifuged at $18\,000 \times g$, the supernatant decanted off and the cells dried to constant weight in a vacuum chamber. Density was calculated as the dry weight of cells per ml of bacterial culture i.e. mg/ml (dry wt.).

Viable cell counts were made by preparing serial dilutions of the bacterial suspension in PRAS 1/4 strength Ringer solution and counting bacterial colonies after growth in WCAB agar under anaerobic conditions (pour plate technique).

5. Methods—Azo reduction experiments

5.1. Quantitative analysis of azo reduction

Azo bond reduction was monitored by spectrophotometric analysis, following reduction of the highly coloured azo bond chromophore, with a Cecil CE 207 spectrophotometer (Cecil Instruments, Cambridge, UK).

Calibration graphs of absorbance versus concentration were constructed from solutions of azo dye in appropriate medium (WCAB or phosphate buffer) and measured against the appropriate reference medium. Beer–Lambert calibration plots were obtained using 1-cm UV cells for each dye-medium combination used.

5.2. Azo reduction in WCAB (method I)

B. fragilis cells were harvested from 100 ml of gently shaken 14-h WCAB culture by centrifugation at $18\,000 \times g$ for 15 min and resuspending the pellet in 100 ml of pre-reduced anaerobically sterile 0.06 M phosphate buffer (pH 7.4). All procedures were carried out aseptically under oxygen-free nitrogen. In an anaerobic chamber (Miller Howe Ltd., Watlington, Oxford, UK), 8 ml of the *B. fragilis* suspension was added to 2-ml aliquots of dye solution (final azo concentration 30 μM) then sealed under an atmosphere of oxygen-free nitrogen. The sealed vessels were incubated at 37°C over a period of up to 8 h and at timed intervals a sample was opened, the contents vortexed for 10 s and the suspension centrifuged at $18\,000 \times g$ for 10 min, in order to obtain a clear supernatant for analysis.

The reduction of amaranth, Orange II and tartrazine by *B. fragilis* was monitored by following the decrease in absorbance at the λ_{max} of each substrate (520 nm; 484 nm and 430 nm, respectively) against a blank comprising the supernatant obtained under identical conditions incubated with water instead of dye solution. Controls comprised dye in phosphate buffer with no bacteria.

5.3. Modified assay method (method II)

Anaerobic conditions were maintained during an experiment by passing a flow of nitrogen through the bacterial suspensions or control solutions. Test bacterial cultures were prepared as previously described. Pre-reduced anaerobically sterile WCAB (100 ml) in a 100-ml multi-dose vial was inoculated with 1 ml of a *B. fragilis* culture in CMM and incubated for 14 h at 37°C. The *B. fragilis* cells were harvested from the 14-h WCAB cultures by centrifuging at $18\,000 \times g$ and resuspended in 100 ml 0.06 M phosphate buffer, pH 7.4. Each 100-ml multi-dose vial was fastened securely with a rubber septum and crimped aluminium seal. To initiate an experiment, 1 ml of the azo substrate solution was injected through the septa to give a final azo concentration of approximately 30 μM. Reaction vials were maintained at 37°C in a water bath and agitated by

continuous bubbling with oxygen-free nitrogen gas. Samples for analysis (1.5 ml) were withdrawn via cannulae (Portex Ltd., Kent, UK) at timed intervals, placed into Eppendorf tubes and the cells sedimented using a bench centrifuge (11 500 × *g*) to yield a clear supernatant for spectrophotometric analysis. A vial containing *B. fragilis* suspension with no added dye was used as a spectrophotometric blank for each time point. Controls were comprised of azo substrate in phosphate buffer incubated and maintained under the same conditions.

5.4. Effect upon azo reductase activity of the redox mediator benzyl viologen

Using method II, the effect upon azo reductase activity of addition of benzyl viologen to the *B. fragilis* suspension was observed. Benzyl viologen was injected prior to the addition of the azo substrate, to give a final concentration of 5 μM. The concentration of 4,4′-dihydroxyazobenzene (azo substrate) in the reaction mixture was 30 μM. The time course of the experiment was reduced to 4 h to reflect the faster reduction of 4,4′-dihydroxyazobenzene compared to the food dyes. A calibration graph of 4,4′-dihydroxyazobenzene concentration versus absorbance (λ_{max} 360 nm) was constructed with the azo substrate dissolved in 0.06 M phosphate buffer, pH 7.4, and phosphate buffer as reference. In the experiment, the test suspensions were measured against a blank suspension of bacteria in phosphate buffer and benzyl viologen with no 4,4′-dihydroxyazobenzene being present.

5.5. Azo reduction at pH 6.4 and pH 7.4

Method II was used to investigate whether any difference in azo reduction rate by *Bacteroides fragilis* would be effected by a change in phosphate buffer suspension medium from pH 7.4 to pH 6.4.

5.6. Azo reduction under aerobic conditions

For this experiment, the procedure outlined in method II was used, except that the test bacterial suspensions were bubbled with air instead of oxygen-free nitrogen gas.

5.7. Increased azo reduction with increasing bacterial cell concentration

The contents of up to three 100-ml vials of *B. fragilis* test suspension were combined in order to investigate the effect of increasing bacterial cell density on extent of azo reduction after 90 min (method II was used).

6. Results

Bacteroides fragilis was found to reduce all three azo dyes, although reduction of each substrate occurred at different rates (Fig. 2). Amaranth ($E_{1/2}$ −568 mV) was reduced at the fastest rate of 0.30 mol l^{-1} h^{-1}, followed by Orange II ($E_{1/2}$ −648 mV) at 0.2 mol l^{-1} h^{-1} and finally tartrazine ($E_{1/2}$ −700 mV) at 0.08 mol l^{-1} h^{-1}. In contrast, none of the azo dyes were reduced when no bacteria were present with WCAB.

The rate of reduction of the azo dyes in the presence of *B. fragilis* under anaerobic conditions

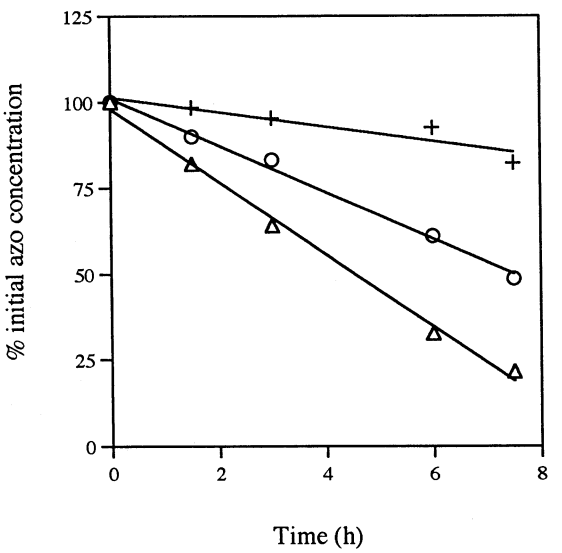

Fig. 2. Reduction of various azo dyes by *Bacteroides fragilis*. (△) Amaranth; (○) Orange II; (+) Tartrazine. Each time point represents one discrete sample.

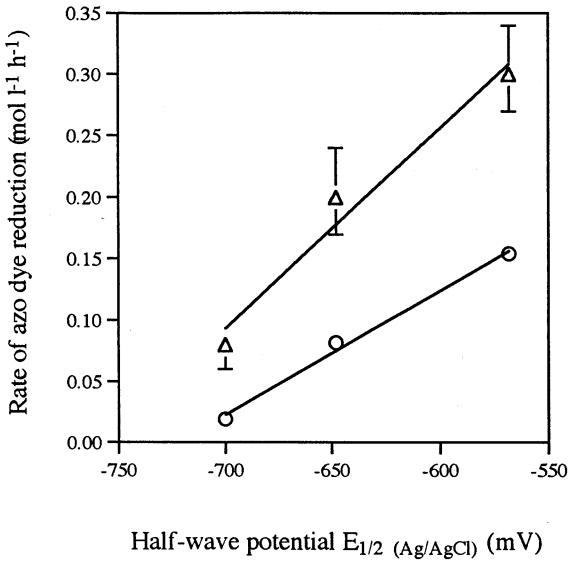

Fig. 3. Rate of azo dye reduction as a function of the $E_{1/2\ (Ag/AgCl)}$ of the dyes amaranth, Orange II and tartrazine, for two anaerobic bacteria. (△) *B. fragilis* ($n = 3$, ± range); (○) *E. limosum* ($n = 1$).

was plotted as a function of the half-wave potentials of the dyes, determined electrochemically as described in Section 3.2. It was found that the rate of reduction of the azo dyes increased with increasing (more positive) half-wave potential, amaranth having an $E_{1/2}$ of −568 mV, Orange II −648 mV and tartrazine −700 mV (Fig. 3). A similar trend was obtained when the dyes were incubated with the organism *Eubacterium limosum*, although the rates of reduction of all the dyes were less when *E. limosum* rather than *B. fragilis* was employed.

Under the experimental conditions described in method II, the rate of reduction of 4,4′-dihydroxyazobenzene monitored over 6 h was determined to be 0.75 mol l^{-1} h^{-1} (Fig. 4). The half-wave potential ($E_{1/2}$) obtained for the polarographic reduction of this compound was −470 mV. When the reduction rates of 4,4′-dihydroxyazobenzene and the azo dyes were compared, it was seen that there was a linear relationship between the logarithm of rate of azo reduction and the half-wave potential (Fig. 5).

The redox mediator benzyl viologen was shown to increase the reduction of 4,4′-dihydroxyazobenzene when present in a suspension of *B. fragilis*, but showed no azo reductase activity without the bacteria (Fig. 4). Altering the pH from 7.4 to 6.4 had no effect on the rate of reduction (Fig. 6).

The *B. fragilis* suspensions, when aerated, did not reduce 4,4′-dihydroxyazobenzene (Fig. 6). However, when the air was replaced with oxygen-free nitrogen, azo reductase activity was restored and occurred at a rate similar to that observed under conditions of continuous anaerobicity (mean pH 7.4; Figs. 4 and 6).

As the concentration of *B. fragilis* cells in the suspension increased from 1 mg/ml to 3 mg/ml dry weight, the number of azo bonds remaining after 90 min correspondingly fell, clearly indicating that azo reductase activity increases with increasing density of bacterial cells (Fig. 7).

7. Discussion

These studies show that the colonic organism *Bacteroides fragilis* is capable of reducing the azo dyes amaranth, Orange II and tartrazine at different rates (Fig. 2) and that there is a relationship

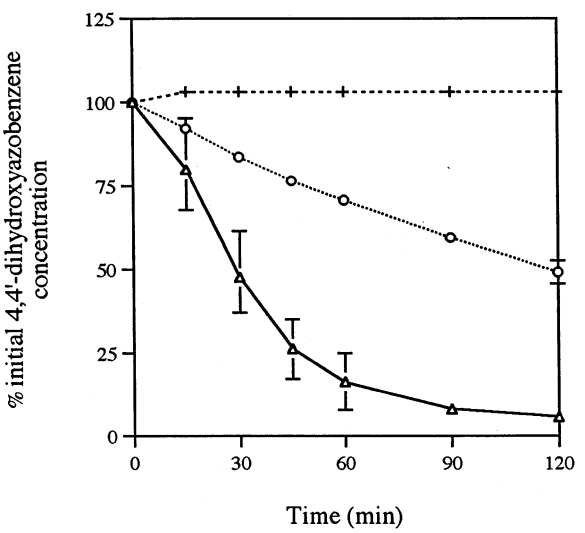

Fig. 4. Enhanced reduction of 4,4′-dihydroxyazobenzene by *B. fragilis* in the presence of benzyl viologen and 0.1 M phosphate buffer, pH 7.4. (△) *B. fragilis* plus benzyl viologen ($n = 3$, ± range); (○) *B. fragilis* alone ($n = 6$, ± S.D.); (+) Benzyl viologen alone.

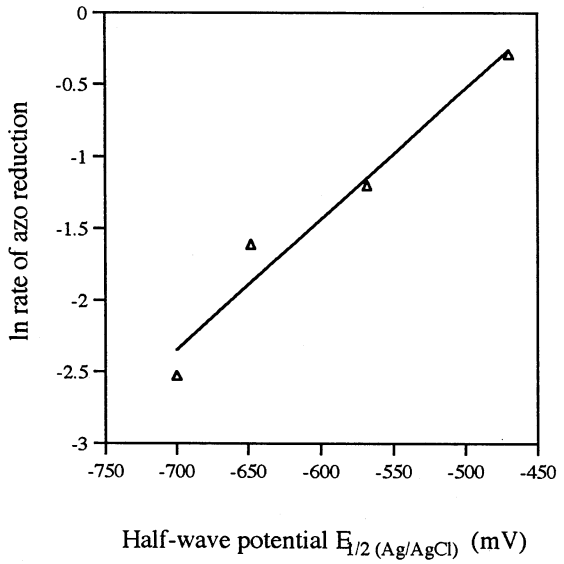

Fig. 5. Logarithm of the rate of reduction of the four azo compounds 4,4′dihydroxyazobenzene, amaranth, Orange II and tartrazine, as a function of the $E_{1/2\ (Ag/AgCl)}$ of the compounds.

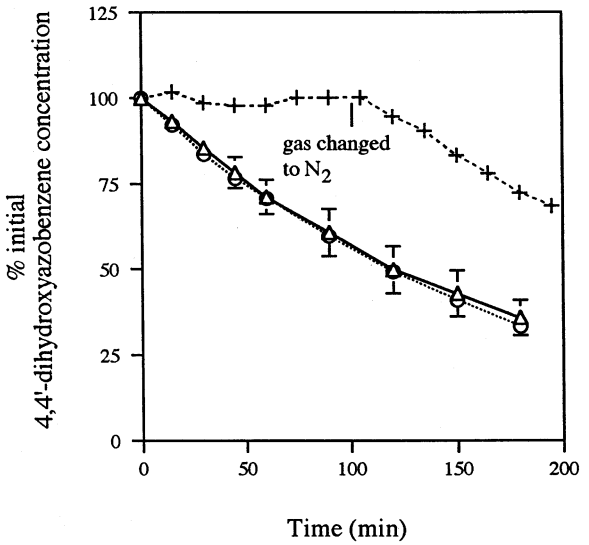

Fig. 6. Reduction of 4,4′-dihydroxyazobenzene by *B. fragilis* in phosphate buffer (0.1 M, pH 6.4 and 7.4) and under conditions of aeration. (△) pH 7.4 ($n = 6$, ± S.D.); (○) pH 6.4 ($n = 6$, ± S.D.); (+) Aeration followed by N_2, pH 7.4 ($n = 2$).

between rate of bacterial reduction by *Bacteroides fragilis* and *Eubacterium limosum* and the redox potential of the azo dyes (Fig. 3). This would be expected since the redox potential is a measure of the ease at which a molecule will accept electrons and be reduced; the more positive the redox potential, the more readily it is reduced. *B. fragilis* reduced the model azo compound 4,4′-dihydroxyazobenzene at a much faster rate (Fig. 4) than the azo dyes and when the rates of reduction were compared, it was observed that a linear relationship exists between the logarithm of the rate of reduction and the half-wave potential of the azo compound (Fig. 5). This relationship was also found by Dubin and Wright (1975) with the organism *Proteus vulgaris*. Zbaida et al. (1994) have recently reported that the reactivity of monosubstituted azo dyes towards azo reduction by rat liver microsomes is also determined by their electron densities and redox potentials.

Bronsted and Kopecek (1992) found that electronic factors appeared to influence ease of reduction of the azo bond in the low molecular weight form, but when the cross-linking agents were incorporated into the polymer the correlation was not repeated. This was suggested to be due to the bromine component of the most readily reduced azo compound causing excessive, uncontrolled polymerisation. Problems such as these require

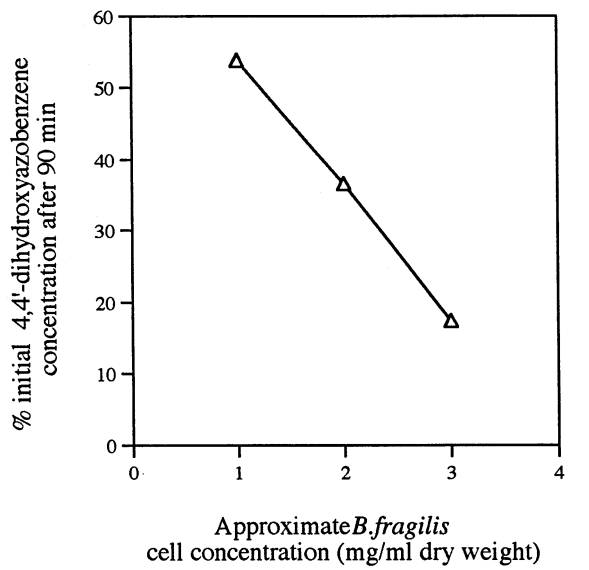

Fig. 7. Reduction of 4,4′-dihydroxyazobenzene after 90 min incubation by increasing concentrations of *B. fragilis* cells.

consideration when designing a cross-linking azo compound, also the length of the spacer chains between the aromatic azo group and the polymer; this length has been found to be very important for ease of azo reduction (Bronsted and Kopecek, 1992; Van den Mooter et al., 1993).

A criticism of the dyes used in the current study is that they are low molecular weight, water soluble dyes and not related to the azo functional groups incorporated into macromolecular polymers for colon-specific drug delivery devices. However, Brown (1981), using a variety of different organisms, demonstrated that polymeric azo dyes were reduced at rates comparable to their low molecular weight analogues. As the polymeric dyes were unlikely to cross the bacterial cell wall, it was concluded that azo reduction occurred extracellularly. It has been postulated by a number of workers (Gingell and Walker, 1971; Bronsted and Kopecek, 1992) that low molecular weight electron carriers such as reduced flavins are responsible for azo reduction, moving across the cell membrane to reduce the azo compound (and thus be oxidised themselves), then returning to the intracellular space to further partake in cellular metabolism, and so on. In aerobic conditions, oxygen would be reduced in preference to azo compounds because of its more positive redox potential, and thus azo reduction would be inhibited. This would explain the observation in Fig. 6 where azo reduction did not occur when the system was bubbled with air rather than nitrogen. However, when the air was subsequently replaced with oxygen-free nitrogen, azo reductase activity was restored, indicating that the inhibition of azo reductase activity of *B. fragilis*, under the aerobic conditions described, was a temporary effect rather than an irreversible inhibition.

The dependence of azo reduction on anaerobic conditions is well documented in many bacterial (Walker, 1970; Rafii and Cerniglia, 1990) and mammalian systems (Levine and Raza, 1988). Some workers (Azad Khan et al., 1983; Zimmerman et al., 1982) have demonstrated azo reductase activity under aerobic conditions; however Azad Khan et al. (1983) reported more efficient cleavage of the azo bond when oxygen was excluded, and most studies on azo reductase activity of colonic bacteria have been performed under anaerobic conditions (Dubin and Wright, 1975; Chung et al., 1978; Brown, 1981; Rafii et al., 1990). Indeed, isolation of the azo reductase enzyme from *Clostridium perfringens* requires very strict anaerobic conditions (Rafii and Cerniglia, 1990). In mammalian cells, Levine and co-workers (Zbaida and Levine, 1991; Levine et al., 1992) have reported two distinct azo reductase systems, an oxygen-sensitive and oxygen-insensitive mechanism.

The requirement for an anaerobic atmosphere is favourable for a colonic delivery system, as premature azo reduction by the low levels of bacteria found higher up the gastrointestinal tract would be prevented by the presence of oxygen. Only as the azo polymer reached the more anaerobic regions of the lower gastrointestinal tract would the azo cross-links be susceptible to reduction and cleavage.

It was found that the reduction of 4,4′-dihydroxyazobenzene could be accelerated by benzyl viologen (Fig. 4), which indicates that a non-enzymatic process is involved in the actual cleavage of the azo bond by the test organism, *Bacteroides fragilis*. This would favour the reduction of large molecular weight polymers by this predominant organism. Chung et al. (1978) showed that benzyl viologen could increase the rate of reduction in whole cell suspensions of *Bacteroides thetaiotaomicron*, and similar results have been reported with cell-free extracts of *Streptococcus faecalis* (Lloyd et al., 1994) and rat caecal contents (Kopeckova and Kopecek, 1994). Others have shown the importance of biological electron carriers such as FMN and FADH in accelerating azo reduction due to their activity as electron shuttles (Rafii et al., 1990; Chung et al., 1978; Gingell and Walker, 1971).

The non-enzymatic reduction of azo compounds by enzymatically generated low molecular weight electron carriers is favourable for a drug delivery system targeting the lower gastrointestinal tract. The low redox potential measured in the colon (Schroder and Johansson, 1973) indicates a very reducing environment constituting a ready availability of extracellular electron carriers. Schacht et al. (1996) recently showed that a reduc-

tive medium consisting of cysteine and sodium sulphide in phosphate buffer, without enzyme, was capable of reducing azo polyamides to the hydrazo form of the azo group, i.e. from –N=N– to –NH–HN–. Schacht et al. (1996) and Kimura et al. (1992) presented evidence that a colour change in azo polymers from yellow to white represented reduction to the hydrazo stage, rather than the fully reduced amine form, which was previously believed to be necessary for polymer degradation and drug release (Bronsted and Kopecek, 1992; Van den Mooter et al., 1994). However, both Schacht et al. (1996) and Kimura et al. (1992) acknowledged that the change in state of the polymer could be sufficient for loss of integrity of the polymer backbone to such an extent as to allow release of encapsulated drug.

The physiological pH of 7.4 has been used for phosphate buffered bacterial suspensions in much of this current, and previous, work on azo reduction (Bronsted and Kopecek, 1992; Chung et al., 1978). However, the pH of the caecal region in humans is reported as 6.4 ± 0.4 (Evans et al., 1988). It was therefore appropriate to determine whether the pH of the suspending medium affected azo reductase activity. Fig. 6 illustrates that, at pH 6.4, azo reduction of 4,4′-dihydroxyazobenzene by *Bacteroides fragilis* proceeded at the same rate as at pH of 7.4, indicating that any fluctuation between these two values in the individual subject would not adversely affect the rate of azo reduction. However, this observation can only be said to be true in these particular circumstances and may not be true for other azo compounds, particularly those with a pK_a between 6.4 and 7.4. Chung et al. (1978) studied the reduction of tartrazine by *Bacteroides thetaiotaomicron* at a variety of different pH values in 0.4 M phosphate buffer and reported an optimal pH range of 7.4–8.5.

The azo reductase activity of the test *B. fragilis* suspension clearly increased with increasing cell density (Fig. 7). The cell density used in these investigations was 1 mg/ml (dry wt.); the density of cells used in many in vitro experiments to investigate reduction of azo compounds and polymers is similarly low. However, it is known that in the colon, bacterial density is much higher than can be achieved in useful model systems and hence the long time periods given for reduction would be expected to be dramatically reduced in in vivo conditions.

The observations on the azo reducing activity of *Bacteroides fragilis* all support the mechanism of azo reduction proposed by Brown (1977), namely that it appears to be a non-specific reaction involving the donation of reducing equivalents to the azo bond by an electron carrier mediator species such as NADH, FMN, benzyl viologen, etc. in which rate determining factors include the redox potential of the mediator (NADH, FMN, benzyl viologen, etc.) in relation to that of the azo dye substrate; specificity of flavoprotein-reducing enzymes with respect to the mediator; steric and electrostatic factors influencing the reduction of the azo dye by the mediator and the permeability of the azo dye substrate to the cellular site of reduction.

No specific azo reductase enzyme has been isolated for any *Bacteroides* species; however various enzymes have been studied for *Proteus vulgaris* (Roxon et al., 1967), *Streptococcus faecalis* (Gingell and Walker, 1971) *Pseudomonas* spp. (Zimmerman et al., 1982) and some anaerobic organisms (Rafii and Cerniglia, 1990, 1993). The azo reductase enzyme from the colonic bacterium *Clostridium perfringens* was found to be a dehydrogenase enzyme synthesised throughout the cytoplasm and secreted without accumulation inside the cell. The similarity of azoreductases from four species of *Clostridium* and a *Eubacterium* species have been evaluated and have indicated a marked homology of the azoreductases from taxonomically distinct genera with regard to their function and antigenicity (Rafii et al., 1992).

8. Conclusions

Bacteroides fragilis suspensions were found to reduce azo dyes at a rate related to the redox potential of the dye. As the redox potential of the azo substrate became more negative, the rate of reduction decreased. 4,4′-Dihydroxyazobenzene ($E_{1/2}$ −470 mV) was reduced at the fastest rate of 0.75 mol l^{-1} h^{-1}, whereas tartrazine ($E_{1/2}$ −700

mV) was reduced at the slowest rate of 0.08 mol l^{-1} h^{-1}.

Reduction of 4,4′-dihydroxyazobenzene was sensitive to oxygen and occurred at a similar rate in WCAB medium and 0.06 M phosphate buffer, whether at pH 7.4 or pH 6.4. The rate of reduction was enhanced by the presence of the redox mediator benzyl viologen and by increasing density of *B. fragilis* cells.

These studies indicate that choice of a polymeric cross-linking azo compound for colon-specific drug delivery should consider the electronic nature of the compound: a high redox potential infers that the azo bond will be readily reduced by an extracellular reducing system in the colon, whereas a cross-linker with a more negative redox potential would be reduced at a slower rate.

Furthermore, these studies have used one of the most common human colonic organisms to show the requirement of azo reduction for an anaerobic atmosphere and its enhancement in the presence of redox mediators and increasing cell density. These requirements are favourable for localising azo reduction in the colon.

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