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Study of some important factors involved in azo derivative reduction by *Clostridium perfringens*

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Abstract

In order to design azo polymers having potential for use as colonic coating materials, the most important factors able to affect the bacterial degradation of azo derivatives were evaluated by a reliable method, using *Clostridium perfringens* ATCC 3626 as colonic bacteria. The azo degradation was followed by recording the decrease of the absorbance of azo dye solutions in phosphate buffer salt (PBS), pH 7.2, containing *Clostridium perfringens*, vs time. The results obtained show that the degradation of azo substrates is linear (zero order), faster in basic media and when a redox mediator, such as riboflavin or benzylviologen, is introduced in the incubation medium. Moreover, the substrate redox potential was shown not to affect significantly the degradation rates. However, when several azo dyes are introduced simultaneously into the incubation medium, the microbial azo reduction occurs sequentially as a function of the substrate redox potential, the substrate having the smallest negative redox potential being degraded first. Finally, when Eudragit® RL/RS films containing an azo dye (amaranth) are incubated with *Clostridium perfringens* or NADPH, the time required to bleach the films decreases dramatically when the percentage of Eudragit® RL in the films increases, as a result of the increase of the film permeability. © 1998 Elsevier Science B.V.

Keywords: Colon-specific drug delivery; Azo reduction; *Clostridium perfringens*; Redox potential; Eudragit®; NADPH

1. Introduction

The site-specific delivery of drugs to the colon has implications in a number of therapeutic areas. These include the topical treatment of colonic diseases (Crohn's disease, ulcerative colitis, carcinomas, microbial infections), the potential oral delivery of proteins, peptides and other labile drugs and the improvement of therapy in diseases susceptible to circadian rhythm such as asthma. Since the discovery of the activation mechanism

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of sulphasalazin (salicylazasulfapyridine) into 5-aminosalicylic acid by the intestinal microflora (mainly localized in the colon), a number of studies have shown the potential of azo polymers for drug targeting to the colon (Saffran et al., 1986; Van den Mooter et al., 1992).

However, the insufficient understanding of the bacterial azo reduction mechanism and the difficulty in obtaining successful colonic delivery systems which need firstly to protect a drug during its transit from mouth to caecum and secondly to afford site-specificity, are obstacles for an efficient design of colonic azo polymers (Lloyd et al., 1994). Although the bacterial metabolism of azo dyes used as food dyes has been extensively investigated with regard to their toxicity, there is still a need for a better understanding of the mechanisms and factors governing the azo dye and polymer degradation by the intestinal flora.

Many bacterial strains (Clostridia, Eubacter, Streptococcus, Bacteroides) of the intestinal microflora are capable of achieving the reduction of azo groups (Chung et al., 1978; Rafii et al., 1990). Although the exact mechanism of the bacterial azo reduction is still a matter of discussion, it seems to involve biological water-soluble electron carriers such as FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), riboflavin, NAD (nicotinamide adenine dinucleotide) or NADP (nicotinamide adenine dinucleotide phosphate). According to some authors, the biological cofactors, acting as electron mediators, are firstly reduced by azo reductase, while the azo compounds act as electron receptors at the end of the electron transport process (Gingell and Walker, 1971; Lloyd et al., 1994). This mechanism suggests that azo reduction is in part mediated by low molecular weight electron carriers rather than by specific azo reductase enzymes. Therefore, a better accessibility of the substrate through polymer films or swollen polymer matrix can be expected.

Among the bacteria of the intestinal microflora, *Clostridium perfringens* possesses the highest azo reduction activity (Rafii et al., 1990). The intracellular localization of *Clostridium perfringens* azo reductase is characterized by a uniform distribution through the cytoplasm. Moreover, the extracellular secretion of azo reductase immediately follows its synthesis without accumulation in the cell (Rafii and Cerniglia, 1993).

The first aim of this study was to design a reliable method for determining bacterial azo-degradation kinetics, using *Clostridium perfringens* ATCC 3626 as test organism. Furthermore, the influence of some experimental factors on the degradation rate was also studied. These experimental factors include the pH of the incubation medium, the ethanol concentration in the medium, the redox potential of azo derivatives, the addition of electron carriers (riboflavin, benzylviologen) and the ability of NADPH to reduce some azo dyes. Finally, the relationship between the Eudragit® RL/RS ratio of films containing an azo dye (amaranth) and the time required for bacteria or NADPH to decolorize these films was studied in order to evaluate the permeability of azo polymer films that could be required to undergo a satisfactory degradation in the colon.

2. Materials and methods

2.1. Preparation and standardization of the inoculum

Clostridium perfringens LMG 10468 (Laboratorium voor Microbiogie en Microbiele genetica Gent, Belgium) was kept at 4°C in thioglycollate–resazurin broth (Bio Mérieux, France) as anaerobic medium. A volume of 0.5 ml of this standing culture was used to inoculate 12 ml of the same broth. After incubation for 20 h at 37°C, the cells were centrifuged at $1595 \times g$ (3000 rpm) for 10 min (Megafuge 1.0, Van der Heyden, Belgium). The bacterial pellets were suspended in 10 ml of PBS buffer, pH 7.2 (2 mM KH_2PO_4; 8 mM $Na_2HPO_4H_2O$; 167 mM NaCl), containing 0.125 mM benzylviologen (Sigma Chemical Co., St. Louis, MO, USA) and 6.3 mM glucose D(+) monohydrate (Merck, Darmstadt, Germany). The PBS buffer was previously deoxygenated by bubbling with oxygen-free nitrogen for 10 min.

The concentration of viable cells in the suspension (inoculum) was evaluated by incubating 10-fold dilutions of the inoculum in the reinforced

Table 1
Physico-chemical characteristics and reduction rates of some azo dyes

Azo dye	λ_{max} (nm)	Incubation medium	K (μmol/ml/h)	$E_{1/2}$ (mV)
Amaranth	522	I	0.74 ± 0.04	-455
Methyl orange	507	I	0.62 ± 0.03	ND
Orange II	483	I	0.7 ± 0.1	-480
Tartrazine	425	I	0.67 ± 0.08	-545
4-Amino-azobenzene	495	II	0.31 ± 0.02	-520
4-Hydroxy-azobenzene	346	II	0.31 ± 0.02	-445
Amaranth	522	II	0.24 ± 0.01	-455

$E_{1/2}$, half-wave potential of the azo substrate. The $E_{1/2}$ values have been determined by the polarographic method, using 0.2 M phosphate buffer, pH 7.2, containing 20% ethanol as solvent. ND, not determined; λ_{max}, wavelength of maximum absorption of the azo dye. The measurements of the absorbances of each azo dye were carried out at its λ_{max}. K, degradation rates of the azo dyes by *Clostridium perfringens* (mean $\pm$ S.D.; at least three determinations). I, PBS, pH 7.2, containing *Clostridium perfringens*; II, PBS, pH 7.2, containing 9% ethanol and *Clostridium perfringens*.

Clostridium agar (RCA) at 37°C under anaerobic conditions (Oxoid Gaspack jar). The bacterial concentration of the inoculum was $3 \pm 1 \times 10^8$ cfu/ml and was close to that of Clostridia in colonic content (1.3×10^9 cfu/g wet weight of faeces) (Ikeda et al., 1994).

2.2. Kinetic studies

2.2.1. Azo reductase activity of Clostridium perfringens

Four ml of PBS buffer, pH 7.2, solutions containing 33 μM azo dyes, 0.125 mM benzylviologen and 6.3 mM glucose D(+) monohydrate, previously deoxygenated by bubbling with oxygen-free nitrogen for 20 min, were distributed into 12-ml wheaton® bottles (Polylabo, France). The bottles were closed with rubber stoppers, left to reach 37°C (10 min), then 0.5 ml of inoculum or PBS buffer (blank) were added to each bottle. After a rapid bubbling with nitrogen, the bottles were sealed with aluminum caps to ensure anaerobiosis and then incubated at 37°C in a horizontal-shaking water bath (GFL 1087, Vel, Belgium), set at 100 shakes/min. At regular time intervals, two bottles were withdrawn from the bath, opened and 0.5 ml of 30% trichloroacetic acid (Sigma Chemical Co., St Louis, MO, USA) aqueous solution was added to stop the reaction. After centrifuging for 10 min at $1595 \times g$, the absorbance of the clear supernatant was measured at the maximum wavelength of absorbance (λ_{max}, Table 1) of the dye. A calibration graph for each dye was carried out by measuring the absorbance of PBS buffer, pH 7.2, solutions containing 3% (w/w) of trichloroacetic acid and known concentrations of the dye. From the calibration graphs, the dye concentration was determined and plotted against time. The rate of degradation (K, the slope of the linear part of the degradation curve) was calculated by linear regression analysis and expressed as micromoles of dye degraded per hour and per ml of inoculum (μmol/h/ml).

All the azo dyes (Fig. 1) used in this study were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA), except tartrazine and orange II (Fluka, Buchs, Switzerland).

2.2.2. Effects of some experimental factors on azo reductase activity

Factors affecting the bacterial azo reduction activity were evaluated by using the same experimental procedure but, in some cases (pH of medium and riboflavin effects, competition of many azo dyes for azo reductase), benzylviologen was intentionally excluded with a view to simplifying the experiment. Amaranth was often used as azo substrate.

The competition of water-soluble azo dyes for azo reductase was evaluated by incubating amaranth, tartrazine, orange II (30 μM of each) together with the inoculum. At regular time intervals, absorbance of supernatant was measured at 425, 483 and 522 nm, which are the

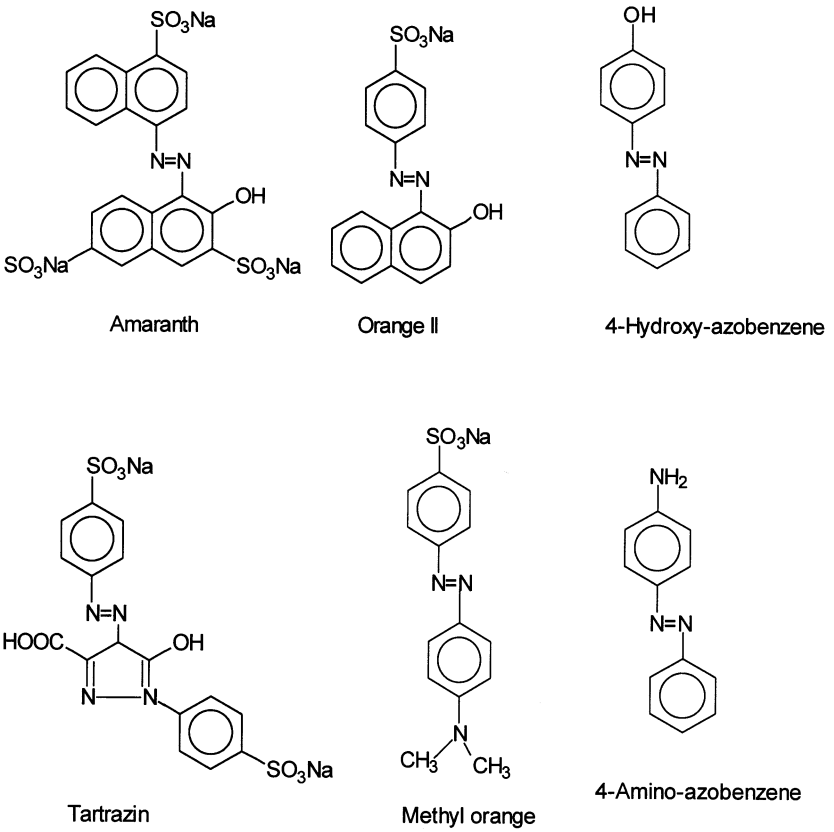

Fig. 1. Chemical structures of azo dyes.

respective λ_{max} values of tartrazine, orange II and amaranth. Therefore, it was possible to calculate the concentration of each dye in the medium as a function of time.

2.2.3. Azo reduction activity of NADPH

The capacity of NADPH to reduce azo dyes was determined under atmospheric conditions (aerobiosis) according to Lloyd et al. (1993). Thirty μM of dye, 0.4 mM benzylviologen and the NADPH generator system (glucose-6-phosphate (0.83 mM), NADP (0.25 mM), glucose-6-phosphate dehydrogenase (1 IU/ml)) were incubated in PBS buffer, pH 7.2, at 37°C and the absorbance of solutions was measured directly, at regular intervals, at the λ_{max} of the dye.

2.2.4. Film decoloration time

On one hand, 200 g of Eudragit® RS 30D aqueous dispersion (30% w/w of solid content, Rhöm GmbH, Pharma Polymers, Germany), 30 nmol of azo dye (amaranth or methyl orange) and 13 g of triacetin (Eastman Chemical Company, TN, USA) as plasticizer were mixed and diluted to 400 g with water. On the other hand, a similar dispersion of Eudragit® RL 30D (30% w/w of solid content, Rhöm GmbH), was also prepared. From these two dispersions (17.25% w/w solid content), blends containing increasing amounts (0, 5, 10, 15, 20, 25, 30, 50, 75 and 100% w/w) of Eudragit® RL in the Eudragit® RS were prepared, and 5 g of each blend were cast in petri dishes (60×15 mm). The plates were covered with funnels in order to prevent a

too rapid evaporation rate. After complete evaporation of water (24 h at 60°C), the films were cured for 48 h at 40°C, removed from the plates and stored in a desiccator until use. The absorbance was determined at 522 nm to evaluate the distribution of amaranth in the films. Assays of different samples taken from different parts of films gave almost constant results: absorbance = 0.220 ± 0.006 (mean $\pm$ S.D., $n = 6$). Blank films without azo dye were prepared under the same conditions. Rectangular pieces of film (about 250 μm in thickness), weighing 45–55 mg, were incubated at 37°C (100 shakes/min) in 4.5 ml of PBS buffer containing 0.125 mM benzylviologen, 6.3 mM glucose and 0.5 ml of inoculum under anaerobiosis conditions or in the presence of the NADPH generator system. At timed intervals, the films were visually examined, compared to blank film and the time required for their total bleaching was recorded.

2.3. Determination of the half-wave potential ($E_{1/2}$)

The solutions of azo dyes (10 mM) in 0.2 M phosphate buffer, pH 7.2, containing 20% ethanol, were swept at a rate of 5 mV/s at 21°C, using a Polarographic analyzer 174 PAR, with a Ag/AgCl electrode as reference. The half-wave potentials $E_{1/2}$ (Table 1), determined from the polarograph, were taken as redox potentials. Twenty percent ethanol in phosphate buffer was employed as solvent because, with certain azo dyes, it was impossible to prepare 10 mM of dye solutions with buffer containing a lower percentage of ethanol. Since we wished to establish a classification of the different azo dyes as a function of their redox potential, it was necessary to use the same experimental conditions for the redox potential determination.

2.4. Statistical analysis

Statistical analysis of the experimental results was performed using, either a two-tailed unpaired or paired Student t-test or analysis of variance (ANOVA) at the significance level $\alpha = 5\%$.

3. Results and discussion

3.1. Azo reductase activity of Clostridium perfringens

The degradation of amaranth and methyl orange by *Clostridium perfringens* vs the incubation time is shown in Fig. 2. It can be seen that, after an initial latency phase, the concentration of azo compounds decreases linearly as a function of the incubation time, indicating that the azo reduction is a zero-order phenomenon.

Moreover, the linearity of the degradation was confirmed by studying the influence of the dye concentration on the degradation rate (not shown). No change of the degradation rate could be observed when the initial concentration of amaranth was doubled.

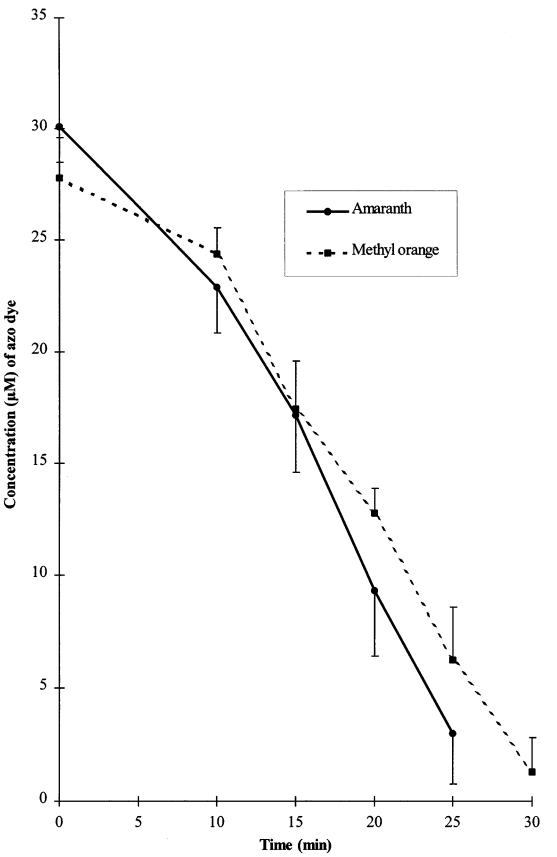

Fig. 2. Reduction of amaranth and methyl orange by *Clostridium perfringens* (mean $\pm$ S.D.; $n = 4$).

The linearity of the azo degradation by intestinal bacteria has been demonstrated also in the literature for *Streptococcus faecalis* (Walker et al., 1971), *Proteus vulgaris* (Dubin and Wright, 1975), *Bacteroides fragilis* (Bragger et al., 1993) and faecal microflora (Kopeckova et al., 1994).

The degradation rates of amaranth and methyl orange by *Clostridium perfringens*, determined by linear regression from the linear part of the degradation curves, were 0.74 ± 0.04 and 0.62 ± 0.03 μmol/ml/h, respectively (not significantly different, unpaired Student t-test, $p > 0.05$). These values are quite similar to those obtained by other workers using faecal microflora as azo reduction medium (1.01 ± 0.3 (Ikeda et al., 1994) and 1.0 ± 0.3 (Kopeckova et al., 1994) μmol/g/h). This method, which can be used for comparative studies between species, is relatively easy, reproducible and therefore can be useful for the in vitro evaluation of azo dye and azo polymer degradation by the intestinal microflora.

3.2. Effects of some experimental factors on azo reductase activity

The influence of the most important experimental parameters on the azo reductase activity are now discussed for a better understanding of the bacterial azo reduction mechanism.

When degradation studies were performed under aerobiosis conditions, the azo reduction proceeds generally at a very slow rate. The inhibition effect of oxygen on bacterial azo reduction can result either from its toxicity for anaerobic bacteria or from a direct inhibition of enzyme or, finally, from the fact that oxygen is a better electron acceptor than azo derivatives.

The influence of redox mediators on the degradation rate of amaranth by *Clostridium perfringens* is shown in Fig. 3. As can be seen, the degradation rate of amaranth is substantially increased in the presence of both riboflavin and benzylviologen. Indeed, redox mediators, which are characterized by a redox potential ranging from -200 to -350 mV, can act as electron shuttles between azo reductase and azo derivatives, and thereby increase the degradation rate (Brown, 1981).

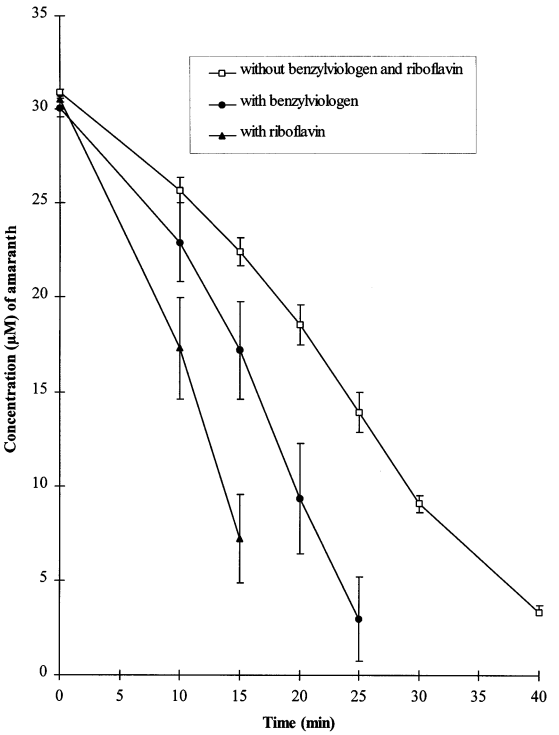

Fig. 3. Effect of 125 μM benzylviologen or riboflavin on the reduction of amaranth by *Clostridium perfringens* (mean $\pm$ S.D.; $n = 3$).

The incorporation of ethanol into the incubation media is often required in order to improve the solubility of slightly water-soluble azo derivatives. Fig. 4 shows the influence of the incorporation of increasing amounts of ethanol on the degradation kinetics of amaranth. A dramatic decrease of azo reduction occurs when the ethanol concentration in the incubation media is increased; the degradation rate is already reduced by about 70% in presence of only 9% v/v of ethanol in the incubation media. Therefore, the impact of organic solvents, such as ethanol, used to improve the solubility of the substrates in the evaluation of azo polymer degradation can be disastrous because of their toxicity or protein denaturing effect.

Some characteristics of azo derivatives such as the half-wave potentials ($E_{1/2}$) and the degradation rates (K) are shown in Table 1. The experiments were carried out in PBS buffer, pH 7.2, for

the water-soluble derivatives (amaranth, methyl orange, orange II, tartrazine) and in PBS, pH 7.2, containing 9% v/v ethanol for other compounds (4-amino-azobenzene, 4-hydroxy-azobenzene). From these results, it appears that no relationship can be established between $E_{1/2}$ and K values. On the contrary, when the same incubation medium is used (medium I or II, Table 1), all the azo derivatives tested are degraded linearly at approximately the same rate (not statistically different, ANOVA, $p > 0.05$), so that the degradation rate appears to be independent of both the redox potential and the structure of the azo dye.

However, these findings are in contradiction with other results reported in the literature with other bacteria (*Proteus vulgaris* (Dubin and Wright, 1975), *Bacteroide fragilis* (Bragger et al., 1993)) for which it appears that the azo reduction rate is increased as the $E_{1/2}$ value of the azo dye increases bearing in mind the negativity of the redox potential.

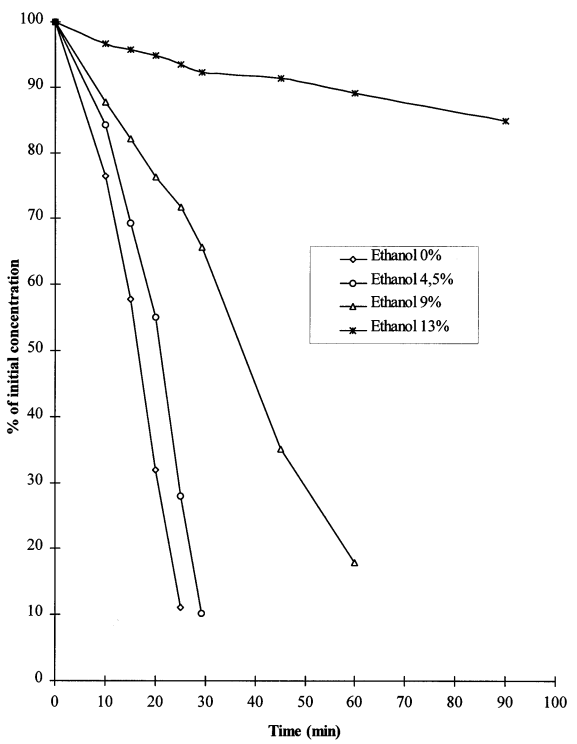

Fig. 4. Effect of ethanol on the reduction of amaranth by *Clostridium perfringens* ($n = 3$).

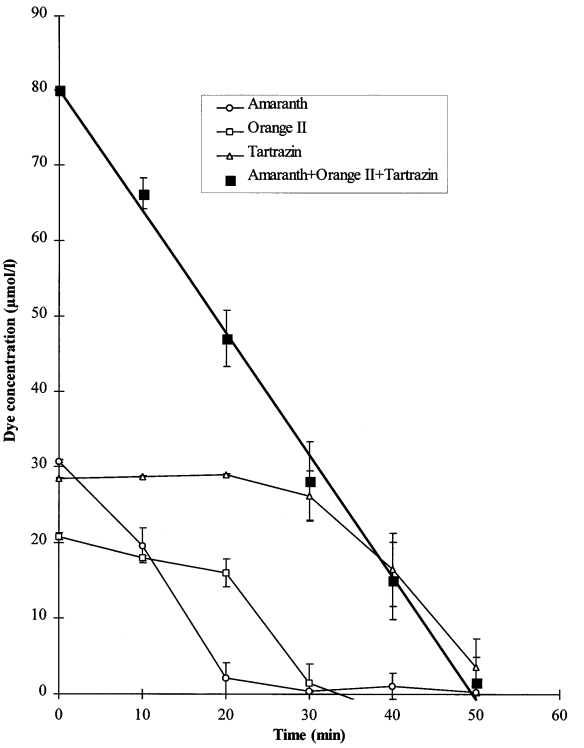

Fig. 5. Reduction curves of three azo dyes (amaranth, orange II and tartrazine) incubated together with *Clostridium perfringens*. Individual (open symbols) and total (filled symbols) reduction curves (mean ± S.D.; $n = 3$).

Fig. 5 shows the degradation results obtained for three water-soluble azo dyes (amaranth, orange II and tartrazine), having different redox potentials, incubated together with *Clostridium perfringens*. This study shows that, after a lag time, the individual (open symbol) and total (filled symbol, obtained by addition of the three individual values at each time interval) degradation of the azo dyes occurs at practically the same rate. On the other hand, the concomitant presence of the three azo dyes in the medium induces degradation processes with different lag times whose duration is as much longer as the negative redox potential of the azo dye is high. From the individual degradation curves (open symbols), it can indeed be observed that amaranth, characterized by the smallest negative redox potential (-455 mV), is degraded first, followed by Orange II (-480 mV) and, finally, by tartrazine which has the highest negative redox potential (-545 mV).

The degradation process occurs exactly as if the azo reductase were able to distinguish between the azo dyes present concurrently in the medium according to their redox potential. An alternative explanation is that the dye which is more readily reducible (smallest negative redox potential) by the cofactors at the end of the electron donor chain is more likely to be reduced first, ahead of compounds that are more difficult to reduce (highest negative redox potential). Anyway, redox potential remains an important parameter which has to be taken into account for the azo polymer design despite the fact that, as shown above, the degradation rate was independent of it.

The degradation rate of amaranth was evaluated at different pH values in order to determine the optimal pH range for azo degradation by *Clostridium perfringens*, and even to underscore how much the variation of pH along the colon (mean $\pm$ SD: from 6.37 ± 0.58 in the right colon ($n = 66$) to 7.04 ± 0.67 in the left colon ($n = 55$)) (Evans et al., 1988) can affect the azo reduction rate. Fig. 6 shows that the azo reductase activity increases dramatically from pH 5.4 to pH 7, but no significant variation (ANOVA, $p > 0.05$) of the degradation rates could be found between pH 7 and pH 9.5. The azo reduction activity of *Clostridium perfringens* is therefore optimal in neutral and alkaline pH media. This seems to be a general feature of the bacterial azo reduction (Chung and Stevens, 1993).

3.3. Azo reduction activity of NADPH

The degradation of amaranth by the NADPH generator system is shown in Fig. 7. As can be observed, the reduction of amaranth by NADPH seems to be a first-order reaction with respect to the dye concentration. Obviously, if one of the substances contributing in the NADPH generator system (NADP, G6P, G6PD) is missing, the reduction of amaranth cannot be observed because of the absence of NADPH generation in the incubation media. In addition, the amaranth degradation rate is faster when benzylviologen is added to the incubation medium and no inhibiting effect of oxygen has been observed (aerobiosis). Similar results have been already reported by Lloyd et al. (1993).

Surprisingly, the NADPH generator system was not able to reduce the other azo dyes tested (methyl orange, orange II, tartrazine, 4-aminoazobenzene, 4-hydroxy-azobenzene). Therefore, in contrast with the degradation tests using isolated colonic microorganisms, the NADPH generator system is not able to provide a universal simple assay method for assessing the reduction process of novel azo derivatives in future studies.

3.4. Film decoloration time

The insoluble but readily permeable Eudragit® RL30D can be mixed in all proportions with the less-permeable Eudragit® RS30D in order to obtain films of graded permeability. Indeed, these two film-forming polymers are often combined to formulate pH-independent, permeable oral sustained-release coatings (Amighi, 1995; Amighi

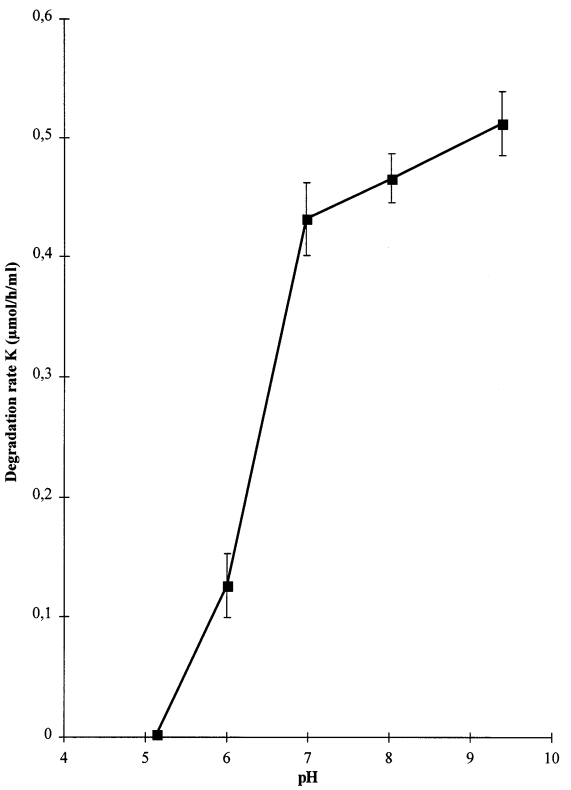

Fig. 6. Effect of pH variations (5.1–9.4) on the amaranth reduction rate by *Clostridium perfringens* (mean $\pm$ S.D.; $n = 4$).

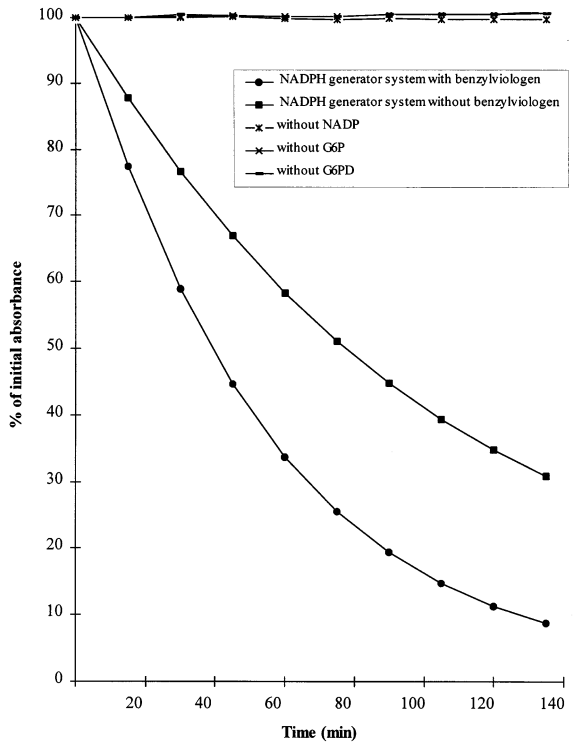

Fig. 7. Reduction of amaranth by the NADPH generator system; BZV, benzylviologen.

and Moës, 1995). Thus, it is worth using them for estimating the permeability required for an azo polymer film to be accessible to the bacteria cofactors or enzymes.

Rectangular pieces of film containing azo dyes were incubated with *Clostridium perfringens* under anaerobiosis, or in the presence of the NADPH generator system, until their total bleaching was observed. No diffusion of the dye out of the films and no bleaching were detected when colored films were incubated in the same conditions without bacteria and NADPH for up to 7 days.

Fig. 8 depicts the times required for bacteria and NADPH to completely bleach the Eudragit® RS/RL films containing amaranth. As can be observed, the time required to bleach the films decreases dramatically when the percentage of Eudragit® RL in the film increases. Moreover, only the films containing more than 10% of Eudragit® RL can be decolorized within 24 h. Therefore, for efficient use, the permeability of azo polymer films should be at least greater than that of Eudragit® RS/RL film containing 10% RL. In earlier works, it has been demonstrated that the bacterial azo degradation rates of azo polymer films were increased with swelling and increasing permeability (Van den Mooter et al., 1994a,b).

It has been also noted that the films containing methyl orange were decolorized by bacteria but not by NADPH. This result was expected since, as shown above, NADPH alone is not able to reduce methyl orange. Moreover, no significant difference could be found (paired Student t-test, $p > 0.05$) between the times taken by bacteria and NADPH to decolorize the films incorporating amaranth. This observation suggests that the molecular sizes of bacteria cofactors acting as electron donors to the azo derivatives could be close to those of NADPH. According to Gingell

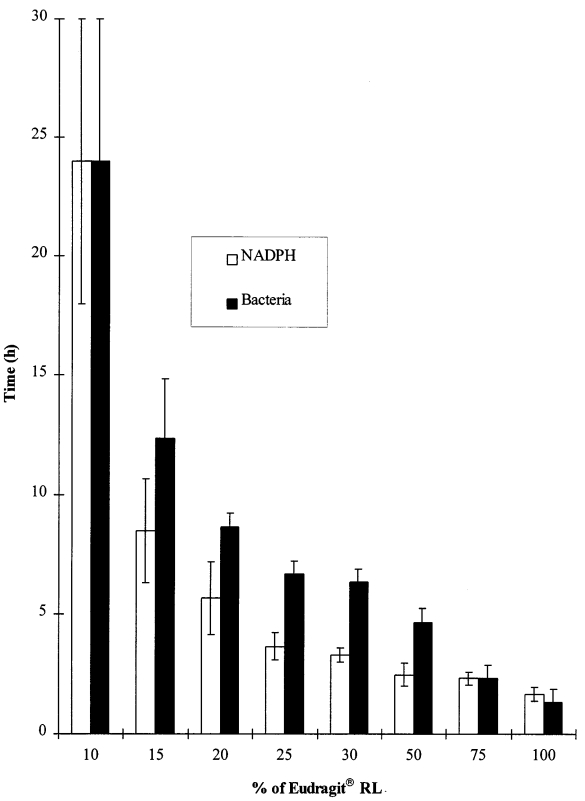

Fig. 8. Time required for NADPH and *Clostridium perfringens* to decolorize the Eudragit® RL/RS films incorporating amaranth (mean ± S.D.; $n = 4$).

and Walker (1971), these cofactors could be the water-soluble flavins ($FADH_2$, $FMNH_2$).

4. Conclusion

This work provides not only interesting data for an optimal design of azo polymers intended to be used as colonic coating materials, but also confirms the validity of the azo polymer approach for colonic drug delivery systems. Indeed, an azo polymer film which would possess a permeability close to that of a Eudragit® RL/RS film containing 10% of Eudragit® RL could protect sufficiently the drug cores during the transit from mouth to caecum and undergo a significant degradation in the colon by the colonic flora.

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References

Amighi, K., 1995. Etude de l'influence des paramètres de formulation, de fabrication et de conservation sur les propriétés de formes orales multiunitaires à libération prolongée, enrobées à l'aide des dispersions aqueuses de polymères acryliques. Thèse de doctorat, Université Libre de Bruxelles.

Amighi, K., Moës, A.J., 1995. Evaluation of thermal and film forming properties of acrylic aqueous polymer dispersion blends: Application to the formulation of sustained-release film-coated theophylline pellets. Drug Dev. Ind. Pharm. 21, 2355–2369.

Bragger, J., Lloyd, A.W., Marriott, C., Bloomfield, S.F., Martin, G.P., Phillips, J., 1993. Redox potential as a factor influencing azo-reduction. Proc. Ann. UKaps Conf. 2, 56.

Brown, J.P., 1981. Reduction of polymeric azo and nitro dyes by intestinal bacteria. Appl. Environ. Microbiol. 41, 1283–1286.

Chung, K.-T., Fulk, G.E., Egan, M., 1978. Reduction of azo dyes by intestinal anaerobes. Appl. Environ. Microbiol. 35, 558–562.

Chung, K.-T., Stevens, S.E. Jr., 1993. Degradation of azo dyes by environmental microorganisms and helminths. Environ. Toxicol. Chem. 12, 2121–2132.

Dubin, P., Wright, K.L., 1975. Reduction of azo food dyes in cultures of *Proteus vulgaris*. Xenobiotica 5, 563–571.

Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Hardcastle, J.D., 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29, 1035–1041.

Gingell, R., Walker, R., 1971. Mechanisms of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. Xenobiotica 1, 231–239.

Ikeda, N., Saito, Y., Shimizu, J., Ochi, A., Mizutani, J., Watabe, J., 1994. Variations in concentrations of bacterial metabolites, enzyme activities, moisture, pH and bacterial composition between and within individuals in faeces of seven healthy adults. J. Appl. Bacteriol. 77, 185–194.

Kopeckova, P., Rathi, R., Takada, S., Rihova, B., Berenson, M.M., Kopecek, J., 1994. Bioadhesive *N*-(2-hydroxypropyl) methacrylamide copolymers for colon-specific drug delivery. J. Control. Release 28, 211–222.

Lloyd, W.A., Hodges, N.A., Soozandehfar, S.H., Martin, G.P., 1993. Azo reduction is mediated by enzymatically generated low molecular weight electron carriers. J. Pharm. Pharmacol. 45, 21P.

Lloyd, W.A., Martin, G.P., Soozandehfar, S.H., 1994. Azopolymers: a means of colon specific drug delivery?. Int. J. Pharm. 106, 255–260.

Rafii, F., Cerniglia, C.E., 1993. Localization of the azoreductase of *Clostridium perfringens* by immuno-electron microscopy. Curr. Microbiol. 27, 143–145.

Rafii, F., Franklin, W., Cerniglia, C.E., 1990. Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. Appl. Environ. Microbiol. 56, 2146–2151.

Saffran, M., Kumar, G.S., Savariar, C., Burnham, J.C., Williams, F., Neckers, D.C., 1986. A new approach to the oral administration of insulin and other peptide drugs. Science 233, 1081–1084.

Van den Mooter, G., Samyn, C., Kinget, R., 1992. Azo polymers for colon-specific drug delivery. Int. J. Pharm. 87, 37–46.

Van den Mooter, G., Samyn, C., Kinget, R., 1994a. Characterization of colon-specific azo polymers: A study of the swelling properties and the permeability of isolated polymers films. Int. J. Pharm. 111, 127–136.

Van den Mooter, G., Samyn, C., Kinget, R., 1994b. The relation between swelling proprieties and enzymatic degradation of azo polymers designed for colon-specific drug delivery. Pharm. Res. 11, 1737–1741.

Walker, R., Gingell, R., Murrells, D.F., 1971. Mechanisms of azo reduction by *Streptococcus faecalis*. I. Optimization of assay conditions. Xenobiotica 1, 221–229.

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