Bacterial Reduction of Azo Compounds as a Model Reaction for the Degradation of Azo-Containing Polyurethane by the Action of Intestinal Flora

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(Received October 26, 1995)

Two types of model compounds containing an azo aromatic group were synthesized, and their reduction kinetics in an anaerobic culture of intestinal flora was investigated for insight into the degradation mechanism of the azo-containing polyurethane which can be specifically degraded by the action of azo reductases which are released by intestinal floras. A water-soluble azo compound, 3,3′-azodibenzenemethanol (ADM), was readily reduced into its hydrazo form and then to the amine form, 3-aminobenzenemethanol (ABM). The reduction rate from azo to hydrazo was much faster than that from hydrazo to amine. In the case of a water-insoluble azo compound, 3,3′-azodibenzenemethanol bis(*N*-phenylcarbamate) (ADM-PC), in which the hydroxy groups of ADM were capped by *N*-phenylcarbamoyl groups, the reduction was stopped in the first step, and only the hydrazo form was produced. These data indicated that the azo-containing polyurethane which is hydrophobic in nature should be degraded by the azo-hydrazo reduction of the azo groups without the chain breakage occurring.

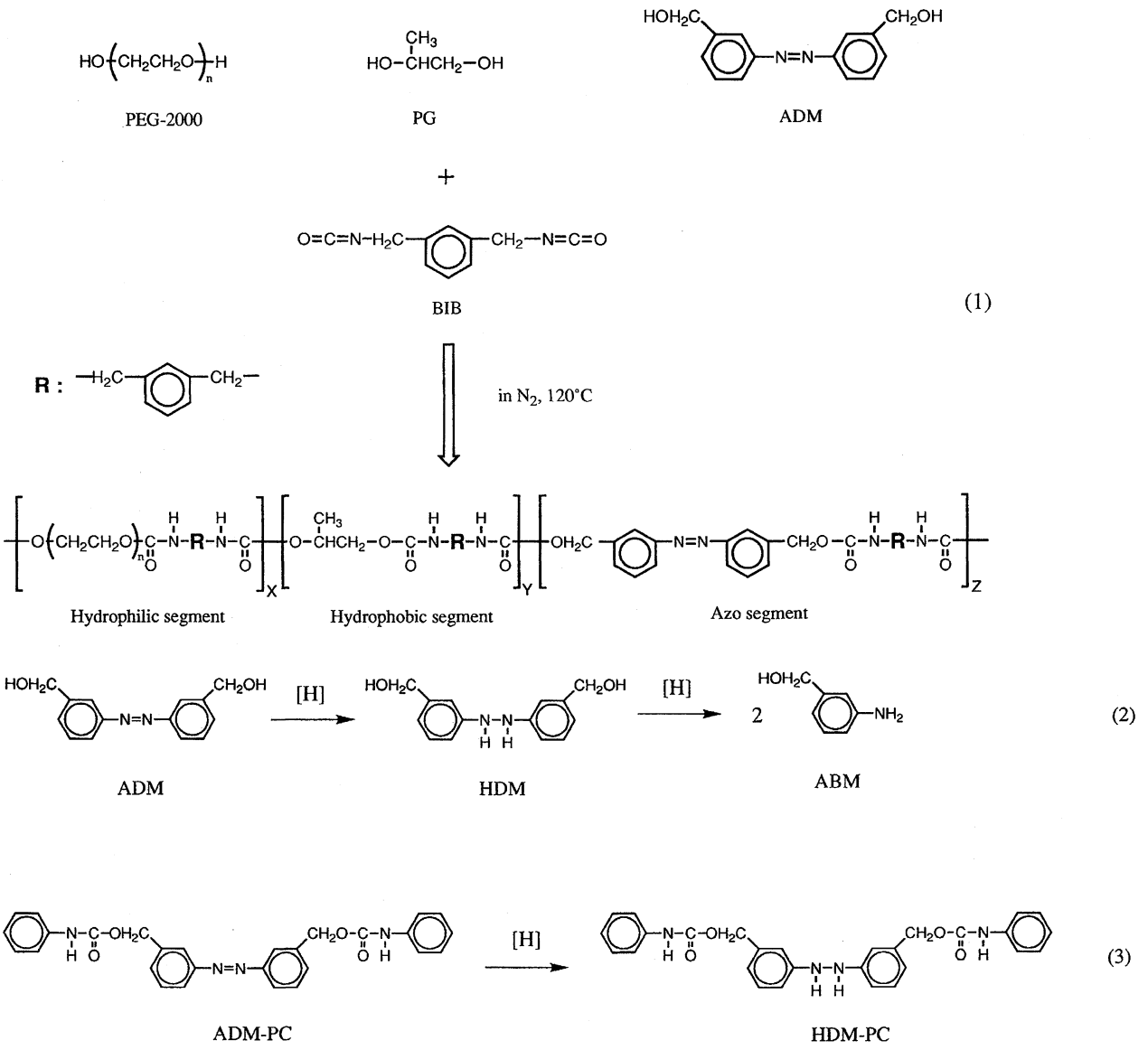
A new colon-specific drug delivery system has recently been proposed by Saffran et al.[1,2] In their system, a hydrophilic vinyl polymer crosslinked by an azo aromatic group was utilized as the coating material of drug pellets and capsules. It was thought that the crosslinks of the polymer coat can be loosened in the large intestine by reduction of the azo group to amine to release the drug incorporated. More recently, we have developed a segmented polyurethane containing azo aromatic groups in the main chain.[3] This polymer is not only degradable by the action of intestinal flora, but also soluble in common solvents and more readily applicable as the coating material of drug. By use of this polymer, we have succeeded in establishing the colon-specific drug delivery.[4] The synthesis of this polyurethane was performed by reaction of 1,3-bis(isocyanatomethyl)benzene (BIB) with a mixture of 3,3′-azodibenzenemethanol (ADM), polyethylene glycol (PEG; $M_n = 2000$ and propylene glycol (PG) (see Eq. 1 of Scheme 1). Its degradability can be readily controlled by changing the segment compositions, i.e., hydrophilic (X: PEG), hydrophobic (Y: PG), and azo-aromatic (Z: ADM) segments. The azo groups of this polymer were found to be reduced to hydrazo groups by the action of intestinal flora, and the degradation of the coating polymer is induced even without cleavage of the azo bonds, because the hydrazo polymer should have decreased cohesive energy and increased absorption of water compared with the original azo polymer.[3—5] However, there has still remained a possibility of the azo groups being reduced to amines, even though their ratio is small. If this would happen, highly toxic oligomers and small molecules comprising amine will be formed and may cause serious effects on the human organisms after absorption. We therefore analyzed the reaction of the azo compounds in the presence of intestinal flora by using several azo compounds as models. This model reaction can give a clear reaction scope for the azo-reduction, together with an insight into the degradation mechanism of the azo-containing polyurethane. In the present paper, the apparent reaction kinetics in an anaerobic culture of intestinal flora was investigated for ADM, which is a starting material of the azo polyurethane (Eq. 2 of Scheme 1), as well as for its urethane-derivative 3,3′-azodibenzenemethanol bis(*N*-phenylcarbamate) (ADM-PC) (Eq.3 of Scheme 1). The hydrazo form of ADM, 3,3′-hydrazodibenzenemethanol (HDM), was also examined as the model compound to study the hydrazo-amine reduction.

Experimental

Materials. All reagents and solvents were purchased from Nacalai Tesque (Kyoto, Japan). ADM was prepared according to the literature method.[6]

The general anaerobic medium (GAM) was supplied from Nissui Pharmaceutical Co., Ltd. (Japan). It is a serum-agar semifluid culture for growing anaerobic microorganisms, containing L-cysteine hydrochloride and sodium thioglycolate as the hydrogen donors.

Measurements. ^{1}H NMR spectra were recorded on a Varian XL-200 (at 200 MHz) spectrometer with tetramethylsilane (TMS) as the internal standard. HPLC was performed on a Shimadzu HPLC



Scheme 1.

system with two LC-10AS pumps, an SPD-10A UV detector (254 nm), and an SCL-10A controller. An ODS column of a COSMOSIL Packedcolumn 5C18-AR (4.6 mm i.d.×150 mm length, Nacalai Tesque, Kyoto, Japan) was used. UV spectra were measured on a Hitachi Model 200-20 double beam spectrophotometer. Mass spectra were obtained at 70 eV using a Hitachi M-80B.

Preparation of HDM. In an appropriate flask 4 g (16 mmol) of ADM, 50 ml of methanol, and 10 ml of an aqueous sodium hydroxide (6.6 mol dm^{-3}) were mixed, and 2.2 g (33 mmol) of zinc dust was added to it. The mixture was refluxed with stirring on a magnetic stirrer at 60 °C for 5 h. Then, the mixture was filtered while it was hot, and the filtered precipitate was washed with a small portion of methanol. The combined filtrate was evaporated to dryness. The residue obtained was washed with water and with diethyl ether in such a way that the product might not be exposed to air. It was finally dried in vacuo. ^{1}H NMR (in acetone-d_6) δ = 4.15 (s, 2H, OH), 4.50 (s, 4H, CH_2), 6.50—6.80, 6.80—6.95 and 7.00—7.15 (m, 10H, C_6H_4 and NH). Because of its easy hydrazo-azo oxidation, the product was contaminated by a trace of ADM even after repeated purification.

Preparation of ADM-PC. 2.0 g (17 mmol) of phenyl isocyanate was added into a solution of 2.0 g (8.3 mmol) of ADM in 20 ml of tetrahydrofuran (THF) at 60 °C. The mixture was stirred for 4 h under a nitrogen atmosphere. At the end of the reaction, 2 ml of ethanol was added to change the unreacted phenyl isocyanate to ethyl phenylcarbamate. The whole mixture was then subjected to the column chromatography (silica gel, hexane/THF (1/1) as the eluent) to isolate ADM-PC. ^{1}H NMR (in *N*,*N*-dimethylformamide-d_7) δ = 5.36 (s, 4H, CH_2), 6.98—7.08, 7.26—7.40, 7.58—7.74 and 7.88—8.00 (m, 18H, C_6H_4 and C_6H_5), 9.87 (s, 2H, NH). MS (EI): Found: *m/z* 480.1789 (M^+). Calcd for $C_{28}H_{24}N_4O_4$: M, 480.1798.

Preparation of 3,3′-Hydrazodibenzenemethanol Bis(*N*-phenylcarbamate) (HDM-PC). In a 200-ml flask 2 g (4.4 mmol) of ADM-PC, 100 ml of THF, and 10 ml of aqueous sodium hydroxide (1.8 mol dm^{-3}) were mixed, and 0.58 g (8.8 mmol) of zinc dust was added to it. The mixture was refluxed with stirring on a magnetic stirrer at 60 °C for 5 h. The mixture was filtered in order to remove the unreacted zinc dust, and the filtrate was evaporated to dryness. Then the residue was subjected to the preparative thin layer chromatography (TLC) (silica gel, hexane/ THF (1/1) as the

developing solution, R_f: 0.63) to isolate HDM-PC and finally dried in vacuo. ^{1}H NMR (in *N*,*N*-dimethylformamide-d_7) $\delta = 5.05$ (s, 4H, CH_2), 6.75—6.90, 6.90—7.20, 7.20—7.40, and 7.55—7.76 (m, 18H, C_6H_4 and C_6H_5), 8.85 (s, 2H, NH). MS (EI): Found: *m/z* 482.1842 (M^+). Calcd for $C_{28}H_{26}N_4O_4$: M, 482.1956.

Preparation of Anaerobic Culture of Intestinal Flora. It is known that more than 70% of human feces are occupied by intestinal floras, both living and dead.[7] Therefore, human feces collected without exposure to air were utilized as the source of the flora.[8] Thus, under a nitrogen atmosphere ca. 1 g of human feces was dispersed in 9 g of GAM, and the mixture was diluted 10 times with GAM to obtain a standard solution of the flora. This was further diluted 200 times with GAM and cultured under anaerobic conditions at 37 °C for 1 d. This culture showed an excellent reductivity to various azo dyes (e.g., Amaranth). It was confirmed that the reductivity was maintained for more than 5 d.

Incubation of the Model Compounds in the Culture. In a culture tube 100 mg of ADM was mixed with 30 ml of the above flora culture. The mixture was incubated at 37 °C under anaerobic conditions for a predetermined period. Then, the mixture was extracted with a 20 ml of ethyl acetate 5 times, and the extracts combined were evaporated to isolate the product. Since ADM isomerized from trans to cis by exposure to light during the extraction, the isolated product was heated at 100 °C for 1 h to make the cis form revert back to the trans form completely. Then, it was analyzed by HPLC. ADM, HDM, and 3-aminobenzenemethanol (ABM) were eluted at the retention times of 8.6, 4.0, 3.1 min, respectively, when detected at 254 nm. Their peak assignment was done by using authentic samples. HDM and ADM-PC were also incubated likewise.

Results and Discussion

Reduction of ADM. Water-soluble ADM was incubated in the anaerobic culture of intestinal flora for 7 d. During this incubation, ADM as well as its reaction product was soluble in the culture without producing any precipitate. Figure 1 shows the reaction profiles. It is known that more than 95% of ADM was consumed in a day, and the amine form ABM gradually increased with incubation time. On the other hand, the hydrazo form HDM rapidly increased, reached a maximum at the incubation time of 6 h, and decreased thereafter. These profiles indicate that ADM was reduced to HDM and then to ABM via a two-step process. We analyzed these reduction steps by the curve fitting method,[9] assuming a first-order kinetics for both steps. The rate constants of the reductions from azo to hydrazo and from hydrazo to amine are denoted by k_1 and k_2, respectively. The fitting curves for the consumption of ADM and for the formations of HDM and ABM are shown by the dotted lines in Fig. 1, for which k_1 and k_2 are determined to be 6.26 d^{-1} and 0.82 d^{-1}, respectively. Each curve is known to fit well with the experimentally obtained plot. Such results support the conclusion that ADM is hydrogenated to ABM via ABM stepwise and that both reactions are in the first-order of the substrates of ADM and HDM. The rate constant k_1 (= 6.26 d^{-1}) was almost eight times higher than k_2 (= 0.82 d^{-1}), although the concentrations of the enzyme and the hydrogen donor involved in these reactions were not known. It should be noted here that the k_1 and k_2 values may be attributed

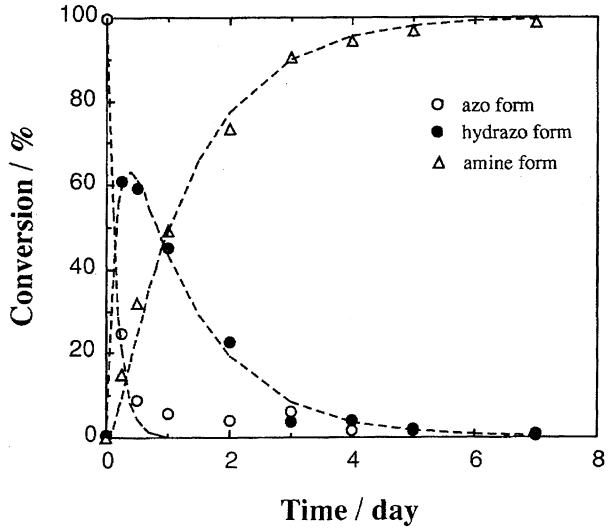


Fig. 1. Azo-hydrazo-amine conversion during the treatment of ADM in flora culture. The dotted lines represent the curves calculated by fitting analysis.

to the reductions catalyzed by different enzymes, because a mixture of various intestinal microfloras was used instead of the discrete reduction enzymes.

Furthermore, the reduction from hydrazo to amine was confirmed by the direct incubation of HDM in the flora culture. A typical reaction profile is shown in Fig. 2. Although the original HDM contained 4% of ADM, the consumption of HDM is known to compete with the formation of ABM. After 7 d, both ADM and the contaminant ADM were reduced completely. The consumption of HDM and the formation of ABM were analyzed by the ordinary first-order kinetics to obtain a straight line between $-\ln C/C_0$ and time t, where C and C_0 denote the concentrations of HDM at time t and t_0, respectively. The rate constant (k_2) was calculated from the slope as 0.52 d^{-1}. This value was slightly lower than that obtained above, but the both values should be taken as com-

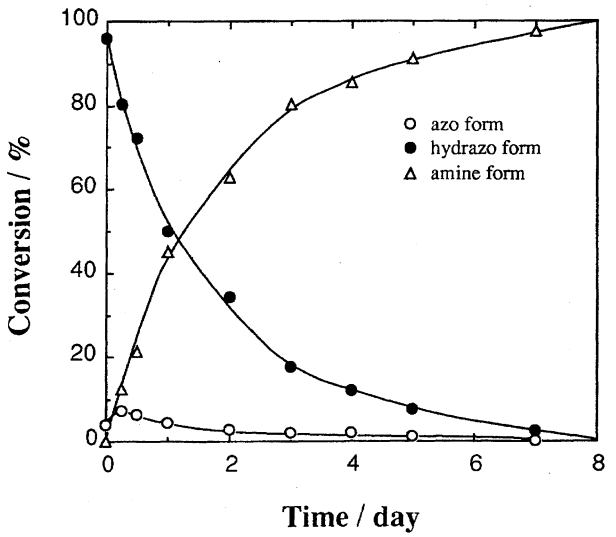


Fig. 2. Hydrazo-amine conversion during the treatment of HDM in flora culture.

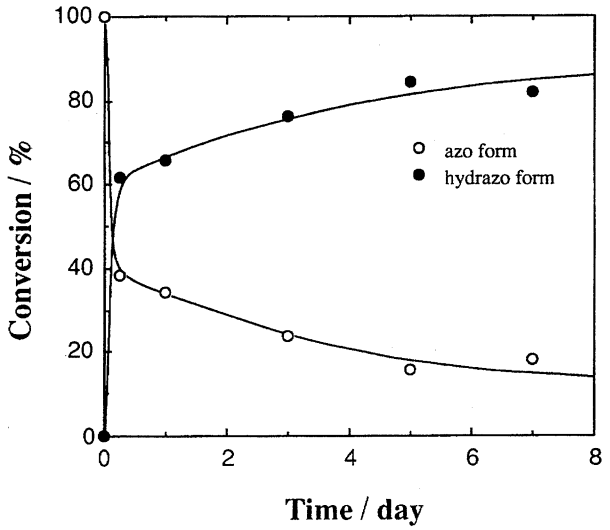


Fig. 3. Azo-hydrazo conversion during the treatment of ADM-PC in flora culture.

parable because the flora culture used for both experiments was taken from different batches. It should be therefore concluded that the hydrophilic azo compound can be reduced to amine form via hydrazo form in the culture of intestinal flora.

Reduction of ADM-PC. Figure 3 shows the similar reaction profile for the reduction of ADM-PC, which is water-insoluble. It is known that ADM-PC was reduced to the hydrazo form without producing the amine form even after 7 d of incubation. The reduction of the azo form was very fast at the initial stage, but slowed down after 1 d. Since both of ADM-PC and its hydrazo form were insoluble in the culture, the reduction should be limited to only the surface area of the compounds in powder form. The apparent rate constant for the initial reduction was calculated as 3.5 d^{-1}, which was half of the value for the first-step reduction of ADM. So the reduction rate of ADM-PC is thought to be almost comparable to that of water-soluble ADM. This suggests that the bacteria can recognize the azo bond for reduction, but cannot reduce the hydrazo bond involved in the precipitate. In other words, a hydrazo compound can be reduced to the amine form only if it is soluble in the aqueous medium.

Reduction Mechanism for the Azo-Containing Polyurethane. In 1962,[10] Radomski and Mellinger investigated the absorption, metabolism, and excretion in rats of the various water-soluble azo-dyes and reported that the azo bond can be reduced to amines by the action of azo reductases of the intestinal microfloras. The present reduction of ADM can be understood as another example of the azo dye. The water-insoluble azo compound ADM-PC, however, gave a different aspect for this azo reduction by intestinal flora, because its reduction was limited to the hydrazo stage. This finding may suggest a possibility that two different enzymes or hydrogen donors may exist for the azo-hydrazo and hydrazo-amine reductions. The azo reductase which catalyzes the azo-hydrazo reduction can act both on water-soluble and water-insoluble substrates, while the reductase which catalyzes the hydrazo-amine reduction can act only on water-soluble hydrazo compound.

We reported on the degradation of the azo-containing polyurethane when incubated in the same flora culture.[3—5] When the polyurethane was incubated in the flora culture, the absorbance at $\lambda_{max} = 320$ nm of the polymer, which was ascribed to the characteristic absorption of the azo aromatic chromophore, decreased with incubation time, while its molecular weight did not change. Moreover, when the polymer incubated in the flora culture was exposed to air, the absorption band at 320 nm gradually increased again and recovered the original absorbance after 20 d of exposure. This was due to the oxidative dehydrogenation of the hydrazo group to the azo group. These results strongly suggested that the azo groups in the polymer were reduced to the hydrazo groups without any the hydrazo-amine conversion occurring during the bacterial treatment. Since the polyurethane was insoluble in water as ADM-PC, only the azo-hydrazo reduction was induced. The degradation of the polyurethane, therefore, should be attributed to the decreased cohesive energy followed by the azo-hydrazo change, by which the polyurethane coat can be mechanically collapsed or water can penetrate into the coat to allow the drug release outside. This reaction mechanism should have a practical merit in that neither toxic oligomer nor amine may be produced in vivo. We are now studying the relation between the segment composition and the degradability.

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