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Reduction of Photohemolytic Activity of Benoxaprofen by β-Cyclodextrin Complexations

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Abstract. Inclusion complexations of benoxaprofen (BXP) with β-cyclodextrin (β-CyD) and heptakis (2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) were studied by the solubility method and CD and ^{1}H-NMR spectroscopy. Both β-CyDs decelerated the photodecarboxylation of BXP, and suppressed the BXP-photosensitized hemolysis, where the inhibitory effect of DM-β-CyD was larger than that of β-CyD. This order was well correlated with the magnitude of the stability constants of BXP-β-CyD complexes. The peroxidation of lipid components in erythrocyte ghosts induced by BXP was also suppressed particularly by DM-β-CyD. The protective effect of β-CyDs on the BXP-induced photohemolysis seems to be due to the suppression in the photochemical reactions of BXP yielding toxic transient species, together with the inhibition in attacks of the transient species to the membrane, through inclusion complexation.

Key words. Benoxaprofen, β-cyclodextrin, di-*O*-methyl-β-cyclodextrin, inclusion complex, photolysis, photohemolysis, lipid peroxidation.

1. Introduction

In a previous study [1], we reported that the photosensitized skin irritation of the antidepressant protriptyline (PTL) was markedly reduced by β-cyclodextrins (β-CyDs), since the formation of highly toxic photoproducts such as the dimer and epoxide of PTL was selectively inhibited through inclusion complexations. In this study, effects of β-CyD and heptakis (2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) on the photosensitized hemolysis of benoxaprofen (2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, BXP), an anti-inflammatory agent, was investigated using human erythrocytes, a simple and reliable model system for estimating phototoxic membrane damage *in vivo*. BXP was chosen as a model drug because its photohemolysis is known to be induced by transient species such as singlet oxygen and BXP radicals [2, 3, 4], which is different from the case of PTL [5].

2. Experimental

2.1. MATERIALS

Benoxaprofen was kindly supplied from Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan). β-CyD and DM-β-CyD were purchased from Nippon Shokuhin Kako, Co., Ltd.

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(Tokyo, Japan) and Toshin Chemical Co., Ltd. (Tokyo, Japan), respectively, and recrystallized twice from water. All other materials and solvents were of analytical reagent grade. Deionized doubly-distilled water was used throughout the study.

2.2. SOLUBILITY STUDIES

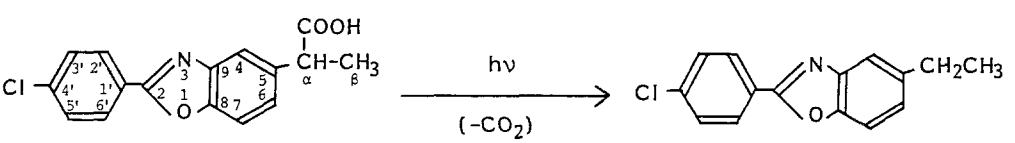
Solubility measurements were carried out according to Higuchi and Connors [6]. Excess amounts of BXP were added to phosphate buffers (pH 2 and 7) containing various concentrations of β-CyDs and were shaken at 25 ± 0.5°C in the dark. After equilibrium was attained (approximately 10 days), an aliquot was centrifuged and pipetted through a cotton filter. The filtrate (0.5 mL) was adequately diluted and analyzed spectrophotometrically at 308 nm. No degradation of BXP was observed under these experimental conditions. Stability constants of inclusion complexes were calculated from the phase solubility diagrams according to the method of Higuchi and Kristiansen [7].

2.3. SPECTROSCOPIC STUDIES

The circular dichroism (CD) spectra was recorded with a Jasco J-50A recording spectropolarimeter (Tokyo, Japan). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were taken on a JEOL JNM-FX 200 spectrometer (Tokyo, Japan), where the concentrations of BXP and β-CyDs in 0.05 N NaOD were 0.02 M. The ^{1}H-chemical shifts were recorded with an accuracy of ± 0.0012 ppm using external tetramethylsilane as a reference.

2.4. PHOTOLYSIS

The light source was a sunlamp (Toshiba FL20SE30, Tokyo, Japan) which emits rays between 290–320 nm, with a maximum at 305 nm (mainly UVB). The irradiance at 305 nm was measured with Radiometer UVR-305/365 (Eisai Co., Tokyo, Japan). The test solutions (6 mL) containing BXP (7.5×10^{-6} M) in phosphate buffers (pH 2 and 7) were photoirradiated at 25°C through a pyrex filter by using a medical UV instrument (Dermaray M-DMR-1, Eisai Co., Tokyo, Japan), under which conditions, no degradation of β-CyDs was observed. At predetermined times, the photoirradiated solutions were acidified with 0.1 N HCl (1 mL) and extracted with 6 mL of ether. After evaporation of the ether phase, the residue was redissolved in methanol (1 mL) and subjected to high-performance liquid chromatography (HPLC) for determinations of BXP and its decarboxylated photoproducts, 2-(4-chlorophenyl)-5-ethylbenzoxazole (DBXP, see Scheme 1), under the following conditions. Pump and detector: Hitachi 635A machine and 638 UV monitor, respectively; column: Whatmann Particil-10 ($4.6\phi \times 250$ mm, USA); mobile phase: acetonitrile/0.1 N acetic acid (3/2); flow rate: 0.8 mL/min; detection: 308 nm; internal standard: diazepam. Thin-layer chromatography (TLC) was performed on silica-gel plate (KIESELGEL 60F 254, Merck, FRG) by using cyclohexane/acetone/0.1 N acetic acid (3/2/1) as developing solvent and an UV lamp (258 and 365 nm) for visualization.



BXP DBXP

Scheme 1.

2.5. PHOTOHEMOLYSIS

Human erythrocytes from freshly drawn blood were supplied by the Kumamoto Prefectural Red Cross Blood Center. Erythrocytes separated by centrifugation ($1000 \times g$ for 10 min) were washed 3 times with isotonic phosphate buffer (pH 7.4) and then resuspended in the buffer solution to give the hematocrit value of 5%. A 0.1 mL of the erythrocyte suspension was added to 4 mL of BXP solution and the mixture was photoirradiated under the same conditions as those described in the photolysis study. At predetermined times, the erythrocyte suspension was centrifuged at $1000 \times g$ for 5 min, and the optical density of the supernatant was measured for hemoglobin at 588 nm. Results were expressed as percent total hemolysis by comparison with a sample of the complete hemolysis in water. Shape changes of the photoirradiated erythrocytes were observed with a scanning electron microscope according to the method of Fujii *et al.* [8].

2.6. LIPID PEROXIDATION

Hemoglobin-free ghost membranes were prepared according to the method of Dodge *et al.* [9] and then resuspended in phosphate buffer (pH 7.4) to obtain a 5% solution. A 0.2 mL of the suspension was added to BXP solution (2.5×10^{-5} M, 4 mL) and photoirradiated for definite times, and an aliquot (1 mL) of the photoirradiated solution was reacted with thiobarbituric acid, according to Buege and Aust [10]. The concentration of lipid peroxide was measured by fluorometric methods and expressed as malondialdehyde (MDA, nmoles/mL ghost membrane) based on the values of tetraethoxypropane as a standard.

3. Results and Discussion

3.1. INCLUSION COMPLEXATION OF BXP WITH β-CyDs

Inclusion complexations of BXP with β-CyDs were studied by the solubility method [6] and CD and ^{1}H-NMR spectroscopy. Figure 1 shows the phase solubility diagrams obtained for BXP with β- and DM-β-CyDs in phosphate buffers of pH 2 and 7 where the substrate exists exclusively in unionized and ionized forms, respectively (pK_a of the carboxylic acid of BXP = 4.4) [11]. The solubility curves for unionized and ionized BXP showed typical A_P- and A_L-type solubility behavior, indicating 1 : 1 and higher order complexations, respectively. The stoichiometries of ionized and unionized BXP-β-CyD complexes were determined to be 1 : 1 and 1 : 2 (guest : host), respectively, by the continuous variation plots [12] of the molar ellipticity changes as shown in Figure 2. Therefore, the linear and ascending plots in Figure 1 were analyzed according to the method of Higuchi and Kristiansen [7] to obtain the stability constants of 1 : 1 and 1 : 2 complexes, and the results are summarized in Table I. The $K_{1:1}$ values were significantly larger than the $K_{1:2}$ values, indicating that the first binding site of unionized BXP to β-CyD cavities is stronger than the secondary one, and the unionized BXP molecule interacts more strongly with β-CyDs than its ionized form. In both pH regions the BXP molecule had a higher affiinity for DM-β-CyD than for the parent β-CyD. The ^{1}H-NMR technique was employed to gain insight into the inclusion mode of the BXP-β-CyD complexes. Table II summarizes the β-CyDs-induced ^{1}H-chemical shifts of BXP in 0.05 N NaOD solution. Upon binding to β-CyDs, all the signals showed downfield shifts and the changes were generally greater in the DM-β-CyD complex than in the β-CyD complex. It is noteworthy that the chemical shift changes were in the

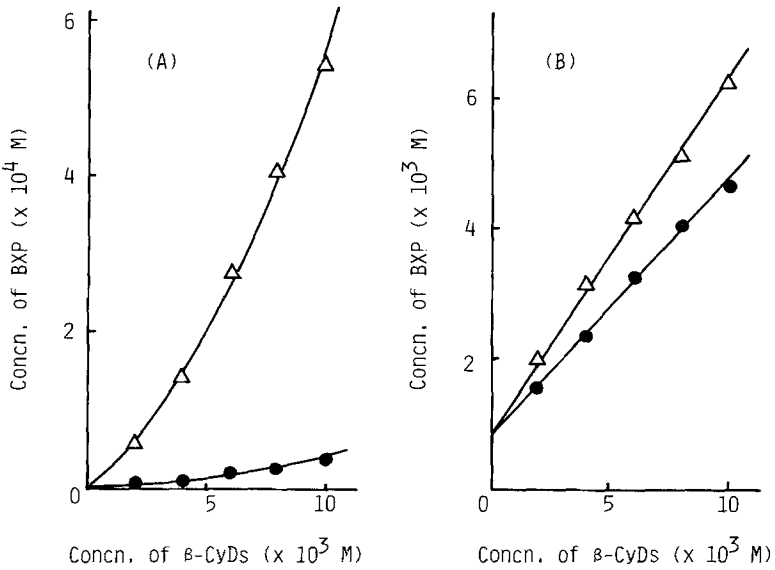


Fig. 1. Phase solubility diagrams of BXP-β-CyD systems in phosphate buffers of pH 2(A) and pH 7(B). ●: β-CyD, △: DM-β-CyD.

order of chlorobenzene (C3′—H, C2′—H) > benzoxazole (C7—H, C6—H, C4—H) > propionate anion (C_β—H) moieties, suggesting that the chlorobenzene moiety of the BXP molecules is deeply included into the β-CyD cavities, particularly DM-β-CyD, to form the 1 : 1 complex in alkaline solution. A similar inclusion mode was also reported for flurbiprofen having a biphenylpropionic acid moiety [13]. In the case of unionized BXP, on the other hand, one more host molecule may be available for the inclusion of the unionized

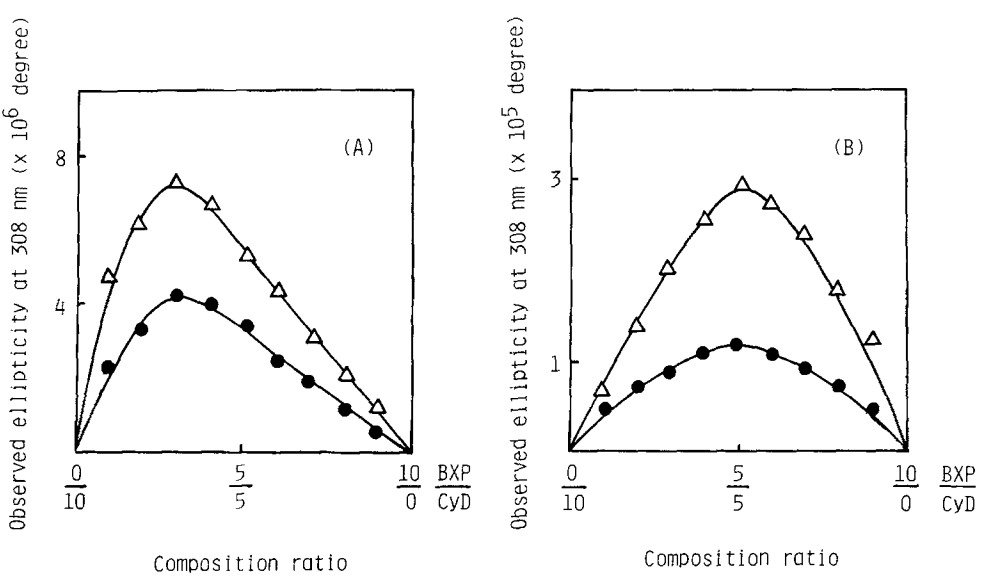


Fig. 2. Continuous variation plots of BXP-β-CyD systems in phosphate buffers of pH 2 (A) and pH 7(B). ●: β-CyD, △: DM-β-CyD.

Table I. Stability constants (K_c, M^{-1}) of BXP-β-CyD complexes in phosphate buffers at 25°C.

System	at pH 2.0		at pH 7.0	
	$K_{1:1}$	$K_{1:2}$	$K_{1:1}$	$K_{1:2}$
BXP-β-CyD	1030	20	700	—
BXP-DM-β-CyD	10100	110	1400	—

propionic acid moiety to form the 1 : 2 complex because this moiety is more hydrophobic than its anionic form, although ^{1}H-NMR spectra of unionized BXP could not be measured accurately due to its low solubility in acidic solution.

3.2. PHOTOLYSIS OF BXP

Figure 3 shows the course of the photodegradation of BXP and the formation of DBXP (Scheme 1) in the absence and presence of β-CyDs in phosphate buffer (pH 2.0). In TLC studies only two spots assigned to BXP ($R_f = 0.41$) and DBXP ($R_f = 0.85$) were observed, and more than 80% of the degradated BXP was converted to DBXP under the experimental conditions. Similar results were obtained even in the presence of β-CyDs, suggesting no appreciable change in the photodegradation pathway of BXP, in contrast to the case of PTL reported previously [1]. It is apparent from Figure 3 that the degradation BXP and the formation of DBXP were slowed by β-CyDs, particularly DM-β-CyD. Figure 4 shows the effect of pH on the first-order photodegradation rate constants (k_{obs}) of BXP in the absence and presence of β-CyDs. The pH-profiles showed sigmoidal curves with a reflection point at pH about 4–5, suggesting that the decarboxylation of BXP is markedly influenced by the ionization of the terminal carboxylic acid. Therefore, the pH-profiles in Figure 4 were analyzed in terms of Eq. (1) on the basis of Scheme 2 to obtain the apparent dissociation constants (K_a) of the terminal carboxylic acid in the presence of β-CyDs.

$$k_{obs} = \frac{k_m \cdot (H^+)^2 + k_i \cdot K_a \cdot (H^+)}{(H^+) + K_a} \quad (1)$$

Table II. ^{1}H-NMR chemical shift changes (ppm) of BXP (2×10^{-2} M) following the binding to β-CyDs (2×10^{-2} M) in 0.05 M NaOD.

Proton	Δδ,[a] ppm	
	β-CyD	DM-β-CyD
C2′,6′—H	0.27	0.35
C3′,5′—H	0.50	0.45
C4—H	0.18	0.24
C6—H	0.19	0.23
C7—H	0.28	0.32
Cβ—H	0.02	0.03

[a] Chemical shift changes are expressed as ($\delta_{CyD} - \delta_0$). See Scheme 1 for carbon numbering of BXP.

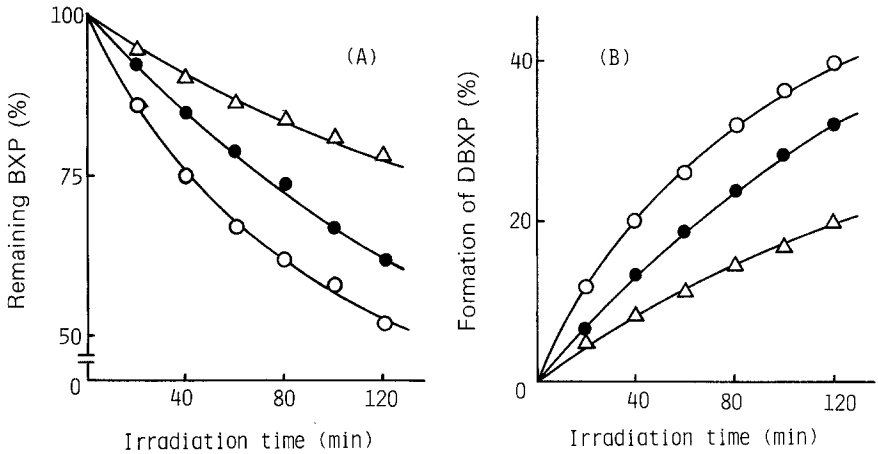


Fig. 3. Effects of β-CyDs (1.0×10^{-2} M) on the remaining BXP (A) and formation of DBXP (B) by photoirradiation of BXP (7.5×10^{-6} M) in 0.1 M phosphate buffer (pH 2.0, $\mu = 0.2$). ○: BXP, ●: BXP + β-CyD, △: BXP + DM-β-CyD. Irradiance is 0.03 mW/cm^2 at 305 nm.

In Eq. (1) k_m and k_i are the degradation rate constants for unionized and ionized BXP, respectively, and (H^+) is the hydrogen ion concentration. The apparent pK_a values refined by a non-linear least squares method are listed in Table III. Unionized BXP was found to be less reactive by a factor of about 12 than its ionized form, and the protolytic dissociation of BXP was suppressed upon binding to β-CyDs; particularly DM-β-CyD. These results suggest that the slowing effect of β-CyDs may be due in part to the inhibition of the ionization of BXP, in addition to the so called microsolvent effect of CyDs [15] because the decarboxylation rate of BXP was retarded in the less polar solvents such as ethanol.

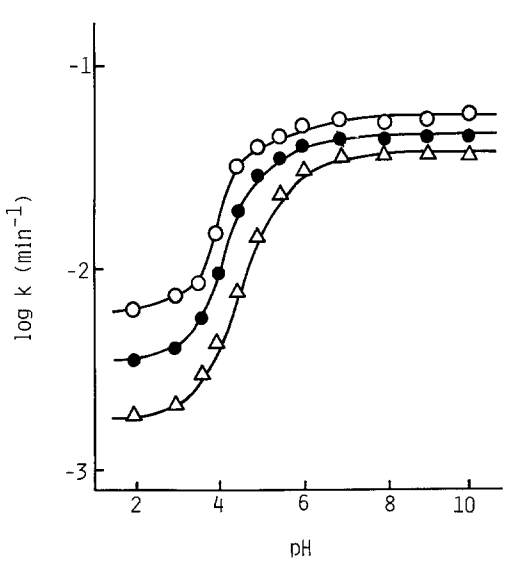
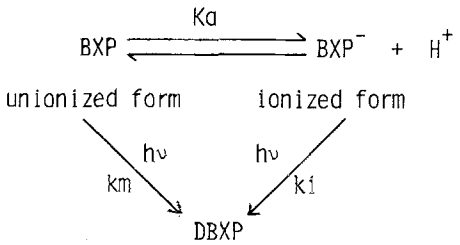


Fig. 4. pH-Rate profiles for photodegradation of BXP (7.5×10^{-6} M) in the absence and presence of β-CyDs (1.0×10^{-2} M). ○: BXP, ●: BXP + β-CyD, △: BXP + DM-β-CyD. Irradiance is 0.03 mW/cm^2 at 305 nm.



Scheme 2.

Table III. Apparent catalytic rate constants (k_m and k_i) and protolytic dissociation constants (pK_a) of BXP (7.5×10^{-6} M) in the absence and presence of β-CyDs (1.0×10^{-2} M).

System	k_m ($\times 10^{-3}$ $min^{-1} M^{-1}$)	k_i ($\times 10^{-2}$ $min^{-1} M^{-1}$)	pK_a
BXP	4.31	5.01	4.27
BXP-β-CyD	3.70	4.50	4.65(4.44)[a]
BXP-DM-β-CyD	2.15	3.75	5.30(5.13)

[a] Values in parenthesis are the pK_a values bound to β-CyDs calculated by the reported method [14].

3.3 PHOTOHEMOLYSIS OF BXP

Photohemolysis of BXP is known to be induced by transient species such as singlet oxygen and BXP radicals formed in the photodegradation reactions [2, 11], rather than by stable phototoxic products observed in the case of PTL [5]. Therefore, the above kinetic results suggest that β-CyDs may reduce the BXP-induced photohemolysis. Figure 5 shows the courses of the photohemolysis induced by BXP in the absence and presence of β-CyDs.

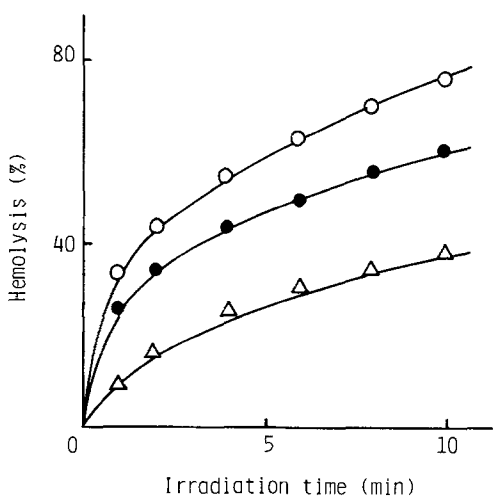


Fig. 5. Effects of β-CyDs (5.0×10^{-4} M) on BXP (2.5×10^{-4} M) photosensitized hemolysis. ○: BXP, ●: BXP + β-CyD, △: BXP + DM-β-CyD. Irradiance is 0.36 mW/cm² at 305 nm.

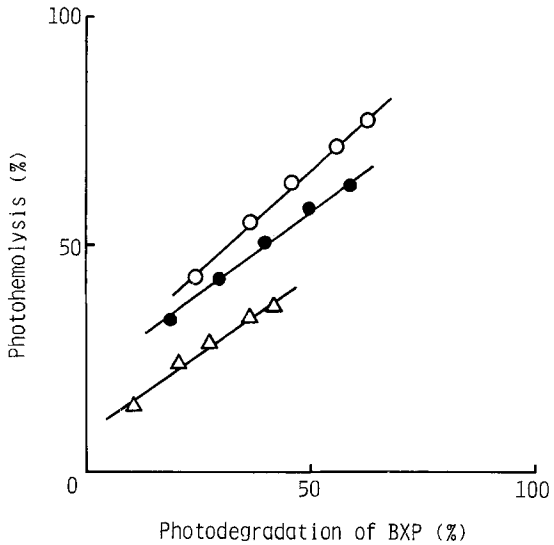


Fig. 6. Relationship between the extent of photohemolysis and photodegradation of BXP in the presence of erythrocytes. ○: BXP, ●: BXP + β-CyD, △: BXP + DM-β-CyD.

The photohemolysis was significantly inhibited by β-CyDs, where the protective effect of DM-β-CyD was larger than that of β-CyD. The BXP-induced shape change of human erythrocytes, i.e. membrane externalization [8], was also prevented by β-CyDs. On the other hand, such hemolysis and shape change were hardly observed when erythrocytes were added to the degradated BXP solution after the photoirradiation. Figure 6 shows the relationship between the extents of hemolysis and the degradation of BXP in the presence of erythrocytes. If the protective effect of β-CyDs resulted merely from the slowing of the degradation of BXP, the plots for the β-CyD systems in Figure 6 should coincide with that

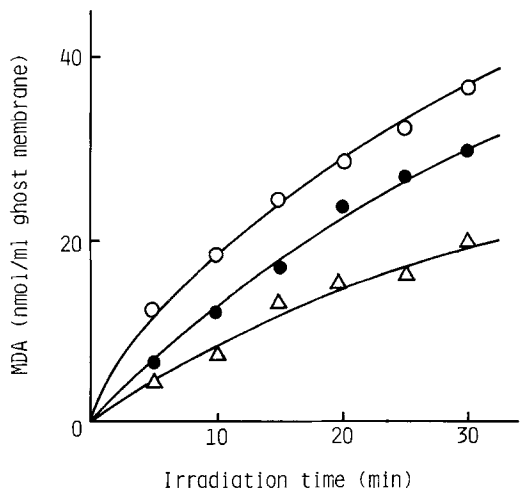


Fig. 7. Effects of β-CyDs (5.0×10^{-4} M) on photosensitized lipid peroxidation in erythrocyte ghosts induced by BXP (2.5×10^{-5} M). ○: BXP, ●: BXP + β-CyD, △: BXP + DM-β-CyD. Irradiance is 1.40 mW/cm^2 at 305 nm.

of the BXP system alone. However, each system gave a different straight line with a correlation coefficient greater than 0.995, and its slope increased in the order of DM-β-CyD (0.70) < β-CyD (0.73) < BXP alone (0.89). These results suggest that attack of the BXP radical and other toxic transient species to the membrane may be also inhibited through the inclusion complexation [16], although at present it is not clear to what extent these species participate in BXP-induced photohemolysis.

Figure 7 shows the course of the BXP-induced photo-oxidation of lipid in erythrocyte ghosts in the absence and presence of β-CyDs. Photoirradiation of BXP solution with the ghost membranes generated amounts of significant lipid peroxides as measured by MDA formation, one of the final products in this process. It is apparent that β-CyDs reduced the peroxidation of lipid, where the inhibitory effect of DM-β-CyD was larger than that of β-CyD. These results suggest that the protective effects of β-CyDs on the BXP-induced photohemolysis may be attributable to the inhibitions not only in the photochemical reaction of BXP yielding toxic transient species such as singlet oxygen but also in their attacks on the membrane, through inclusion complexation [17, 18].

Although, the detailed mechanism should be investigated further, the present data suggest that DM-β-CyD is particulary useful in inhibiting the photodegradation of BXP and alleviating the photosensitized membrane damage caused by BXP.

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