

## Accelerating Effect of Adenine on Riboflavin Sensitized Photooxidation of Mitomycin C

KAZUO UEDA\*

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The effect of adenine on the riboflavin sensitized photooxidation of mitomycin C was investigated. It was found that the photooxidation of mitomycin C is greatly accelerated by the addition of adenine and the linear relationship between the apparent rate constant of the photooxidation and the adenine concentration was obtained with the different slope at different riboflavin concentration. Moreover, the plots of the rate constant against the concentration of riboflavin-adenine molecular complex instead of the adenine concentration was also linear with the essentially same slope at different riboflavin concentrations. In addition, the slope of the Arrhenius plots of the apparent rate constant of the photooxidation was positive in the presence of adenine, whereas negative in the absence of that. These results indicate that the photosensitized oxidation of mitomycin C is accelerated by the riboflavin-adenine molecular complex as a more efficient photosensitizer than riboflavin alone.

Mitomycin C (MC, Fig. 1), an effective anti-cancer agent, can effectively induce the development of  $\lambda$ -phage in lysogenic *Escherichia coli* K-12 ( $\lambda$ ) cells (1). It was reported that the induction of  $\lambda$ -phage formation was suppressed when the lysogenic cells were exposed to visible light at wavelengths between 400 and 500 nm in the presence of MC (2, 3). In the course of studies on the mechanism of the photosuppression, it was found that MC loses the phage-inducing and antibiotic activities on *in vitro* irradiation with visible light in the presence of riboflavin (RF). RF is known as a favorable sensitizer for photoinactivation of various enzymes and antibiotics and for photooxidation of indoleacetic acid and others. MC has an indolequinone ring as shown in Fig. 1. We found that MC is photooxidized by a RF-sensitized reaction and the photooxidation involves singlet oxygen as the reactive species (4). Furthermore, the investigation of the kinetic relation between the photoinactivation and photooxidation of MC in the presence of RF suggested that the photoinactivation

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\*Department of Chemistry, Faculty of Liberal Arts, Nagasaki University, Bunkyo-machi, Nagasaki, Nagasaki 852

tion of MC was caused by its photooxidation( 5 ). On the other hand, it has been reported that the RF-sensitized photoinactivation of yeast alcohol dehydrogenase( 6 ) and the lumiflavin-sensitized photooxidation of indoles( 7, 8 ) are greatly accelerated by the addition of adenine. Based on the fact that mitomycin reductase may be a flavin enzyme( 9 ), it is suggested that flavin moiety of the reductase in *E. coli* cells presumably sensitizes the photoinactivation of MC( 3 ). This suggestion leads us to postulate that adenine appears to play an important role in the RF-sensitized photooxidation of MC in *E. coli* cells.

Therefore, the present author was interested in investigating the effect of adenine on the RF-sensitized photooxidation of MC *in vitro*. It was found that adenine similarly promotes the photooxidation of MC. This report is concerned with the accelerating effect of adenine on the RF-sensitized photooxidation of MC in detail.

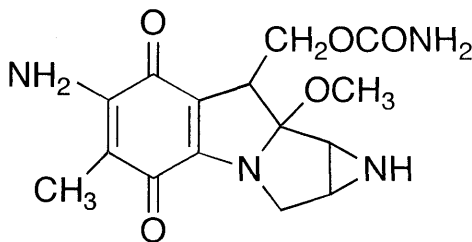


Fig. 1. Structure of mitomycin C(MC).

## MATERIALS AND METHOD

**Materials**—MC was obtained in the form of a mixture with sodium chloride from Kyowa Hakko Kogyo Co., Ltd. and was used after extraction with ethanol to remove the salt. RF was purchased from Nakarai Chemicals Ltd. and purified in a similar manner of literature (10). Adenine and hypoxanthine were obtained from Wako Pure Chemicals Industries Ltd. Distilled and deionized water was used in all experiments.

**Apparatus and Procedure**—The photolysis was carried out as has been described (at above 400 nm and room temperature)( 4 ). The experiments of the temperature dependency of the reaction were only done with aeration in a water bath under illumination. The intensity of the incident light was determined by means of a compensated thermopile (Kipp & Sonen, Delft, Holland).

All the experiments were carried out in a 0.05 M phosphate buffer at pH 7.0. The pH values of sample solutions were measured with a Hitachi-Horiba F-5 pH meter. The solution of MC was stored at 5 °C in a refrigerator and used within a few days after preparation. The solution of RF was kept in a dark place. The concentrations of MC and RF were determined by the absorbance at 362 and 445 nm, respectively. Absorbance and absorption spectra were obtained with a Hitachi model 124 double beam spectrophotometer.

The MC concentration of the reaction mixtures was followed by the photometric method as described in previous papers( 4, 5 ). The apparent rate constants of the photosensitized oxidation of MC were calculated from the initial reaction rate in order to

avoid the effects of photoproducts.

## RESULTS AND DISCUSSION

*Effect of Adenine on the RF-sensitized Photooxidation of MC*—When the aqueous solution containing  $12.5\ \mu\text{M}$  RF and  $9.3\ \mu\text{M}$  MC was irradiated with visible light in the presence ( $12\ \mu\text{M}$ ) or absence of adenine, the photodestruction of MC was greatly accelerated by the addition of adenine as shown in Fig. 2. Figure 2 illustrates the plots of the residual fraction of MC in solutions against the dose of incident light. Judging from TLC of the photoreaction mixtures in the presence or absence of adenine, using n-butanol-acetic acid-water (4 : 1 : 5, volume) as solvent, the photoproducts of MC in the presence of adenine were same as those in the absence of adenine (data not shown). This result indicates that the same photooxidation of MC as that reported previous papers (4, 5) is occurred in the present RF-sensitized photoreaction of MC in the presence of adenine. Both RF and adenine had no effect on the destruction of MC under the dark conditions. Without RF there was no photooxidation of MC in the presence or absence of adenine.

*Effect of Hypoxanthine of the RF-sensitized Photooxidation of MC*—Alternatively, it is expected that the acceleration of this reaction by adenine maybe result from the photoproduct

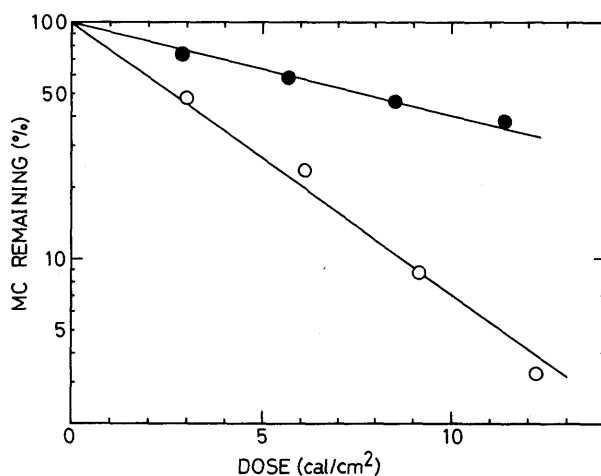


Fig. 2. Effect of adenine on the RF-sensitized photooxidation of MC. A solution containing RF ( $12.5\ \mu\text{M}$ ) and MC ( $9.3\ \mu\text{M}$ ) in a total volume of 3.5 ml was irradiated with visible light at  $22^\circ\text{C}$  in the presence or absence of adenine (○:  $12\ \mu\text{M}$ , ●: none).

of adenine. It is known that hypoxanthine is obtained as the photoproduct of the RF-sensitized photooxidation of adenine but the yield of the product is extremely low (17).

Therefore, we investigated the RF-sensitized photooxidation of MC in the presence of hypoxanthine instead of adenine. Although hypoxanthine also accelerated the RF-sensitized photooxidation of MC, it was found that the accelerating ability of hypoxanthine was lower than that of adenine; the ability of  $83\ \mu\text{M}$  hypoxanthine corresponded to that of about  $13\ \mu\text{M}$  adenine as shown in Fig. 3. The fact indicates that the accelerating effect of adenine cannot be explained by hypoxanthine as a photoproduct of adenine.

*Dependence of the Rate of the RF-sensitized Photooxidation of MC on the Concentration of*

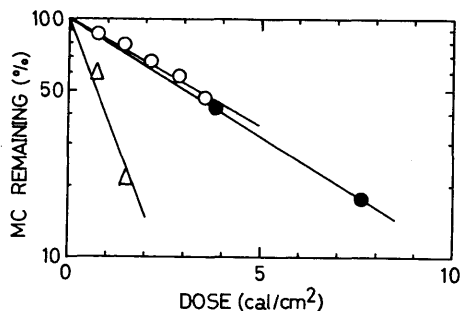


Fig. 3. Effect of hypoxanthine on the RF-sensitized photooxidation of MC. A solution containing RF ( $9.8\ \mu\text{M}$ ) and MC ( $8.2\ \mu\text{M}$ ) in a total volume of 3.5 ml was irradiated with visible light at  $24^\circ\text{C}$  in the presence of purine (○:  $83\ \mu\text{M}$  hypoxanthine, ●:  $13.3\ \mu\text{M}$  adenine, Δ:  $133\ \mu\text{M}$  adenine).

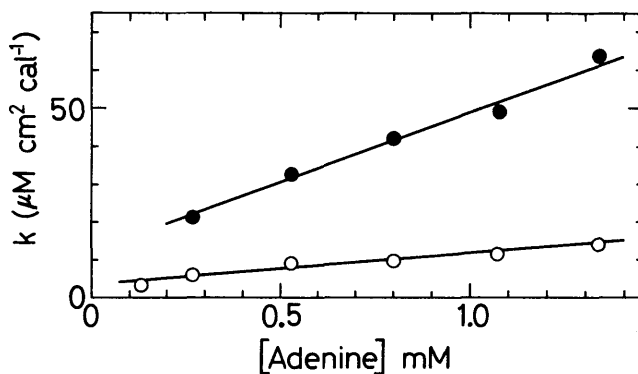


Fig. 4. Dependence of the apparent rate constant of the RF-sensitized photooxidation of MC on the concentration of adenine. A solution containing RF (○:  $9.0\ \mu\text{M}$ , ●:  $45.4\ \mu\text{M}$ ) and MC ( $8.6\ \mu\text{M}$ ) in a total volume of 3.5 ml was irradiated with visible light at  $23^\circ\text{C}$  in the presence of adenine ( $0.13$ – $1.30\ \text{mM}$ ). The intensity of the incident light was  $0.060$ – $0.070\ \text{cal}/\text{cm}^2/\text{min}$ .

*Adenine*—To study the relationship between the apparent rate constant of the RF-sensitized photooxidation of MC and the concentration of adenine, several preliminary kinetic runs were performed at different concentrations of adenine. It was found that the photooxidation of MC obeyed zero-order kinetics when the adenine concentration was higher than about 100  $\mu\text{M}$ , whereas the rate depended on MC concentration when the adenine concentration was lower than about 10  $\mu\text{M}$ . Therefore, measurements were made on above 100  $\mu\text{M}$  adenine to obtain the apparent zero-order rate constants. The concentration of MC was held constant at 8.6  $\mu\text{M}$ , whereas the RF concentration were 9.0 and 45.4  $\mu\text{M}$ , respectively. The plots of the apparent zero-order rate constants of the photooxidation of MC against the adenine concentration are shown in Fig. 4.

It was found that a linear relationship between the apparent zero-order rate constant of the RF-sensitized photooxidation of MC and the concentration of adenine was held as shown in Fig. 4. Moreover, as the RF concentration was changed from 9.0  $\mu\text{M}$  to 45.4  $\mu\text{M}$ , the slope of the plots increased. The apparent zero-order rate constants,  $k$  ( $\mu\text{M cm}^2 \text{ cal}^{-1}$ ), of the photooxidation of MC can be graphically expressed as follows :

$$k_1 = 8.45 \times 10^{-3} [\text{adenine}] + 3.47 \quad (1)$$

$$k_h = 38.47 \times 10^{-3} [\text{adenine}] + 11.65 \quad (2)$$

where  $k_1$  and  $k_h$  are the apparent rate constants at the concentration of 9.0  $\mu\text{M}$  and 45.4  $\mu\text{M}$  RF, respectively, and  $[\text{adenine}]$  is the adenine concentration ( $\mu\text{M}$ ). The intercepts in Eqs. 1 and 2 are the apparent rate constants in the absence of adenine.

It is known that flavins could form molecular complexes with adenine or other purine bases in aqueous solution (11–13) and the RF-adenine complex can be obtained as red-yellowish plate-like crystals (14). In addition, we observed that RF and MC did not form any complex (4). These results suggest that such a large increment of the rate of photooxidation of MC by adenine may be explained by the formation of a RF-adenine complex.

*Relationship between the apparent rate constant of the RF-sensitized photooxidation of MC and the con-*

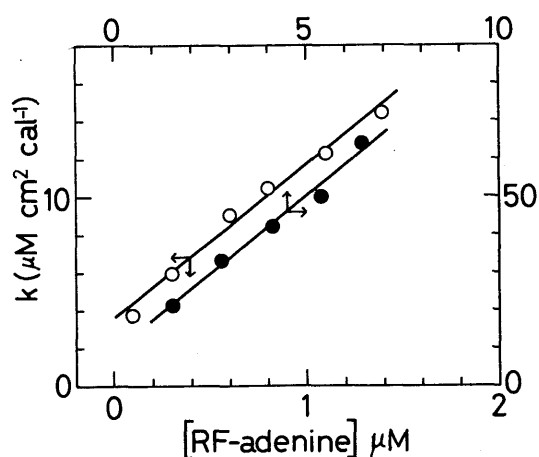


Fig. 5 Relationship between the apparent rate constant of the RF-sensitized photooxidation of MC and the concentration of the RF-adenine complex. Experimental conditions are described in Fig. 4. ○: 9.0  $\mu\text{M}$ , ●: 45.4  $\mu\text{M}$  RF.

*centration of the RF-adenine complex*—Next we investigated the relationship between the concentration of the RF-adenine complex and the apparent rate constant of the photooxidation of MC. Tsibris *et al.* (12). have reported that RF complexed with adenine in aqueous solution and the apparent dissociation constant for 1 : 1 complex formed was calculated as 8 mM at 25°C. Using this value we calculated the concentration of the complex of RF and adenine. The plots of the appaent rate constants of the photooxidation of MC against the calculating concentration of the RF-adenine complex in place of the adenine concentration are shown in Fig. 5.

The apparent zero-order rate constants,  $k_i$  in Eq. 1 and  $k_h$  in Eq. 2, are also expressed using the concentration of the RF-adenine complex in place of the adenine concentration as follows :

$$k_i = 8.91[\text{RF-adenine}] + 3.12 \quad (3)$$

$$k_h = 8.20[\text{RF-adenine}] + 9.28 \quad (4)$$

whrer  $[\text{RF-adenine}]$  is the concentration of the complex of RF and adenine. The intercepts in Eqs. 3 and 4 are also the apparent zero-order rate constants in the absence of adenine. In spite of the different concentration of RF, the slopes of the plots were essentially same ; 8.91 and 8.20  $\text{cm}^2 \text{ cal}^{-1}$ . This indicates that the photooxidation of MC is accelerated by the RF-adenine molecular complex.

*Effect of the RF Concentration on the Photooxidation of MC*—The effect of the RF concentrations on the apparent rate constants of the photooxidation of MC (9.0  $\mu\text{M}$ ) in the presence of adenine (1.33 mM) was investigated. As shown in Fig. 6, the apparent zero-order rate constants of the photooxidation was proportional to the RF concentration up to 20  $\mu\text{M}$ , but the relationship was not linear above 20  $\mu\text{M}$ , because of the effect of self-quenching of RF.

*Temperature Dependency for the Photooxidation of MC*—It was reported that the stability of the RF-adenine molecular complex was decreased with increase in temperature (11, 12). Therefore, if the RF-adenine complex is responsible for the accelerating effect of adenine on the RF-sensitized photooxidation of MC, the rate of the photooxidation will be increased with

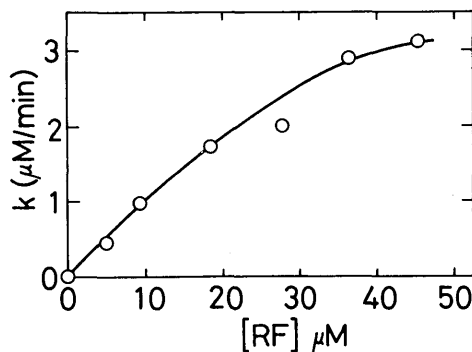


Fig.6. Effect of the RF concentration on the photooxidation of MC in the presence of adenine. A solution containing RF (0 – 45.4  $\mu\text{M}$ ), MC (9.0  $\mu\text{M}$ ), and adenine (1.33 mM), in a total volume of 3.5 ml, was irradiated with visible light at 20°C. The intensity of the incident light was 0.060  $\text{cal}/\text{cm}^2/\text{min}$ .

decrease in temperature. To elucidate the reaction mechanism, the rates of the photooxidation of MC were measured in aqueous solutions with aeration in the presence or absence of adenine at different temperatures. As we would expect, the slopes of the Arrhenius plots of the apparent rate constants of the MC photooxidation are negative in the absence of adenine, whereas slightly positive in the presence of that as shown in Fig. 7. These facts also indicate that the accelerating effect of adenine is accounted for by the formation of a ground-state RF-adenine molecular complex.

From the results described above, it is possible to propose the following reaction mechanism. The RF-sensitized photooxidation of MC in the presence of adenine is greatly accelerated by the RF-adenine molecular complex as the photosensitizer. The excited triplet state of the RF-adenine complex is considered to be a possible intermediate for the photooxidation of MC. There is no other explicit evidence about the triplet state of the complex, but it must be taken into consideration in explaining the accelerating effect of adenine. The excited triplet state of the RF-adenine complex efficiently reacts with ground-state oxygen to form singlet oxygen, which is the reactive species and finally oxidizes MC.

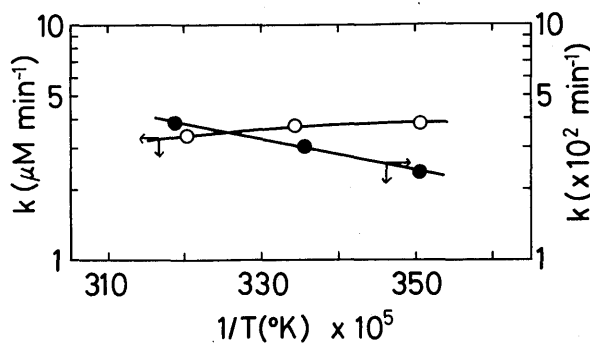


Fig. 7. Temperature dependency for the RF-sensitized photooxidation of MC in the presence or absence of adenine. A solution with aeration containing RF (9.4  $\mu\text{M}$ ), MC (8.0  $\mu\text{M}$ ), and adenine ( $\circ$ : 1.33 mM,  $\bullet$ : none), in a total volume of 3.0 ml, was irradiated with visible light at several temperatures. The intensity of the incident light was 0.39 cal/cm<sup>2</sup>/min.

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