INTERACTIONS OF ANTICANCER QUINONE DRUGS, ACLACINOMYCIN A, ADRIAMYCIN, CARBAZILQUINONE, AND MITOMYCIN C, WITH NADPH-CYTOCHROME P-450 REDUCTASE, XANTHINE OXIDASE AND OXYGEN

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The properties of the interactions of anticancer quinone drugs, aclacinomycin A, adriamycin, carbazilquinone, and mitomycin C with nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase and xanthine oxidase under anaerobic and aerobic conditions were studied. K_m values of NADPH-cytochrome P-450 reductase for these drugs were in the range of 40–227 μM , and that of deflavo xanthine oxidase in the range of 39–over 200 μM . Under anaerobic conditions, when xanthine was used as an electron donor, deflavo xanthine oxidase catalyzed the reductive glycosidic cleavage reaction of anthracyclines and nicotinamide adenine dinucleotide was ineffective as an electron donor. In the electron spin resonance study, the formation of the semiquinone or free radical state of the quinone drugs in both enzyme systems were evidenced. A weak and symmetric signal was obtained from aclacinomycin A, and a symmetric signal from adriamycin was changed into an asymmetric and strong. The hyperfine structure was obtained from carbazilquinone in the oxidase system. In the studies of ultraviolet-visible spectra of the quinone drugs in the reductase system, the spectra of aclacinomycin A and adriamycin were changed to their 7-deoxylaglycones, and the formation of small amounts of the fully reduced form were observed after long incubations. The spectrum of carbazilquinone was changed to the hydroquinone form and mitomycin C was converted into mitosene analogues. Under aerobic conditions, superoxide radicals and hydrogen peroxide were effectively produced in the presence of anticancer quinone drugs in both enzyme systems. The superoxide-dependent hydroxyl radical production, which was measured by ethylene production from methional, was observed in the presence of aclacinomycin A and adriamycin in the deflavo xanthine oxidase system. From these results, the possible reactions in the interactions of anticancer quinone drugs with these enzymes and oxygen are discussed.

Keywords — anticancer quinone drug; aclacinomycin A; adriamycin; carbazilquinone; mitomycin C; NADPH-cytochrome P-450 reductase; xanthine oxidase; superoxide; hydrogen peroxide; hydroxyl radical

INTRODUCTION

In the course of studies on the metabolism of anticancer anthracycline antibiotic, aclacinomycin A (Fig. 1), we have found that nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase (EC 1.6.2.4) catalyzes the reductive glycosidic cleavage reaction, which corresponds to the degradation of anthracycline antibiotics, such as aclacinomycin A, adriamycin, and daunomycin, under anaerobic conditions.^{1,2)} The same reaction is catalyzed by the enzymes which are active on quinone compounds, such as DT-diaphorase (EC 1.6.99.2), xanthine oxidase (EC 1.2.3.2), and mitochondrial nicotinamide adenine dinucleotide (NADH)

dehydrogenase (EC 1.6.99.3).³⁾ We have found that NADPH-cytochrome P-450 reductase and xanthine oxidase catalyze the reductive activation of mitomycin C under anaerobic conditions.⁴⁾ On the other hand, under aerobic conditions, hydrogen peroxide and hydroxyl radicals were produced in the system consisting of NADPH-cytochrome P-450 reductase and anticancer quinone drugs. The hydroxyl radical production, which was measured by the ethylene production from methional, was enhanced by the presence of iron chelate and heme containing proteins.^{3,5,6)} From these studies, the mechanism for the quinone drug metabolism was proposed as the intramolecular electron flow in the semi-

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FIG. 1. Structure of Anticancer Quinone Drugs Tested

quinone form of the quinone drug and, for the production of oxygen related radicals and hydrogen peroxide, the reduction of molecular oxygen by the semiquinone form of the quinone drug and the consequent reactions were suggested.³⁻⁷⁾ In this paper, using aclacinomycin A, adriamycin, carbazilquinone, and mitomycin C, the basic properties of the interactions of anticancer quinone drugs with NADPH-cytochrome P-450 reductase, xanthine oxidase, and oxygen were studied to confirm and extend the previously suggested mechanism. In some xanthine oxidase studies, we used deflavo oxidase because native oxidase produces superoxide and hydroxyl radicals even without the quinone drug.⁸⁾ A preliminary account of these studies was reported.⁹⁾

MATERIALS AND METHODS

Materials — NADPH-cytochrome P-450 reductase was purified from phenobarbital-treated rabbit liver microsomes by the method described by Taniguchi *et al.*¹⁰⁾ The purified enzyme showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the specific activity of cytochrome c reduction was about 28 units/mg of protein. Deflavo xanthine oxidase was prepared by the KI-dialysis method which was described by Kanda *et al.*,¹¹⁾ and the obtained deflavo oxidase usually showed less than 5% of xanthine oxidase activity compared with the original enzyme. Chromatographically purified xanthine

oxidase from butter milk, bovine superoxide dismutase (EC 1.15.1.1), type I, twice-crystallized bovine liver catalase (EC 1.11.1.6), and methional were obtained from Sigma (St. Louis, Mo., U.S.A.). Glucose oxidase (EC 1.1.3.4) was purchased from Boehringer (Mannheim, West Germany). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was obtained from Oriental Yeast Co., Ltd. (Japan). Aclacinomycin A, adriamycin, and carbazilquinone were the kind gifts of Dr. T. Oki (Sanraku-Ocean Co., Ltd., Japan), Dr. F. Arcamone (Farmitalia-Carloerba S.p.A., Italy), and Sankyo Co., Ltd. (Japan), respectively. Daunomycin and mitomycin C were products of Meiji Seika Kaisha, Ltd. (Japan), and Kyowa Hakko Kogyo Co., Ltd. (Japan), respectively. Precoated silica gel 60F₂₅₄ plates for thin-layer chromatography (TLC) were obtained from Merck (West Germany). All other chemicals were analytical grade. The stock solutions of carbazilquinone were prepared by dissolution in dimethylsulfoxide at concentrations of 2–10 mM. For Ultraviolet (UV)-visible spectrophotometric measurement, a Shimadzu UV-200S double beam spectrophotometer was used.

Oxygen Uptake — Oxygen uptake was measured using a Clark-type oxygen electrode. The reaction mixture, in a total volume of 2.0 ml, consisted of: 0.1 M Tris-HCl, pH 7.4; 0.1 mM NADPH; various amounts of anticancer quinone drugs, and 5–14.8 μ g of NADPH-cytochrome P-450 reductase. The reaction was

started by the addition of the reductase. In the case of xanthine oxidase: 50 mM potassium phosphate buffer, pH 7.8; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 0.15 mM xanthine; 0.1 mM anticancer quinone drug, and 31.8 μ g of deflavo xanthine oxidase were used. The reaction was carried out at 30 °C and the oxygen uptake was recorded.

NADPH Consumption — The reaction mixture, in a total volume of 1.0 ml, consisted of: 0.1 M Tris-HCl, pH 7.4; 0.1 mM NADPH; various amounts of anticancer quinone drugs, and 7.4 μ g of NADPH-cytochrome P-450 reductase. The reaction was started by the addition of the reductase at 30 °C and the decrease in the absorbance at 340 nm was recorded.

Quinone Compound Dependent Xanthine Oxidase Activity — The reaction mixture, in a total volume of 1.0 ml, consisted of: 50 mM potassium phosphate buffer, pH 7.8; 0.1 mM EDTA; 0.15 mM xanthine; various amounts of anticancer quinone drugs, and 9.7–19.4 μ g of deflavo xanthine oxidase. The reaction was started by the addition of the enzyme at 30 °C and increase in the absorbance at 292 nm was measured.

Reductive Glycosidic Cleavage Reaction of Anthracyclines — The reaction was carried out under an atmosphere of nitrogen in Thunberg tubes as reported previously.^{1–3)} The reaction mixture consisted of: 50 mM potassium phosphate buffer, pH 7.5; 0.2 mM xanthine or NADH; 0.2 mM aclacinomycin A or adriamycin, and 19.4 μ g of deflavo xanthine oxidase. Xanthine or NADH was placed in the side arm of the vessel before the reaction. The reaction was carried out at 37 °C for 30 min.

Electron Spin Resonance (ESR) Study — The ESR measurements were carried out as reported previously.⁷⁾ In the case of NADPH-cytochrome P-450 reductase, the incubation mixture consisted of: 0.1 M Tris-HCl, pH 7.5; 1 mM NADPH which was placed in the side arm of the Thunberg tube; 0.4 mM aclacinomycin A or adriamycin, and 128 μ g/ml of the reductase. In the case of xanthine oxidase: 50 mM potassium phosphate buffer, pH 7.6; 0.4–1.0 mM anticancer quinone drug; 1.0–1.7 mM xanthine which was placed in the side arm of the vessel, and 667 μ g/ml of the oxidase were used. In the oxidase experiments of aclacinomycin A and adriamycin, the sample solution was prepared under an atmo-

sphere of air and 387 μ g/ml of the oxidase was used. In usual measurements, the modulation amplitude of 0.63 G, microwave power of 10 mW, and time constant of 0.3 s were adopted.

UV-Visible Spectrum — The incubation mixture, in a final volume of 2.0 ml in a Thunberg-type cell, consisted of: 0.1 M Tris-HCl, pH 7.5; 10 μ M NADPH; 40–50 μ M anticancer quinone drug; 6.7 mM $MgCl_2$; 1.5 mM glucose-6-phosphate; 10 μ g glucose-6-phosphate dehydrogenase; 50 mM glucose; 10 μ g glucose oxidase; 10 μ g catalase, and 11.8 μ g of NADPH-cytochrome P-450 reductase which was placed in the side arm of the vessel. In the experiment of aclacinomycin A, $MgCl_2$ was omitted, and in the experiments of carbazilquinone and mitomycin C, 5.92 μ g of the reductase was used. In the case of xanthine oxidase: 50 mM potassium phosphate buffer, pH 7.6; 65 μ M xanthine; 24–25 μ M anticancer quinone drug; 50 mM glucose; 10 μ g glucose oxidase; 10 μ g catalase, and 80 μ g of xanthine oxidase which was placed in the side arm of the vessel, were used. In the experiment of aclacinomycin A, 40 μ g of the oxidase was used. After a cycle of evacuation and filling with nitrogen, glucose oxidase was added, which was followed by two cycles of evacuation and filling with nitrogen. The reaction was started by the addition of the enzyme at room temperature and the spectral changes of the drugs were recorded. After the incubation, anticancer quinone drug and its metabolites in the mixture were extracted and developed on TLC using the solvent system previously described^{1,2)} and chloroform-methanol 10:1 (v/v) for 7-deoxyadriamycinone and carbazilquinone, and ethylacetate-acetone 1:1 (v/v) for mitomycin C. The amounts of the substrate and products were measured using the method which was described in the reductive glycosidic cleavage reaction. The spots of the carbazilquinone and mitomycin C and its metabolites were detected under UV light.

Adrenochrome Formation — In the case of NADPH-cytochrome P-450 reductase, the reaction mixture, in a total volume of 1.0 ml, consisted of: 0.1 M Tris-HCl, pH 7.4; 0.2 mM NADPH; 0.4 mM epinephrine; 0.1 mM anticancer quinone drug, and 2.96 μ g of the reductase. In the case of xanthine oxidase: 50 mM potassium phosphate buffer, pH 7.8; 0.1 mM EDTA; 0.15 mM xanthine; 0.4 mM epinephrine; 0.1 mM anticancer

quinone drug, and 8.2 μg of deflavo xanthine oxidase were employed. In some experiments, 20 μg of superoxide dismutase was added. The reaction was started by the addition of the enzyme at 30 °C and the increase in the absorbance at 480 nm was measured.

Ethylene Production — Ethylene production from methional was measured using a gaschromatography as reported previously.^{5,6)} The reaction mixture consisted of: 50 mM potassium phosphate buffer, pH 7.8; 0.1 mM EDTA; 0.15 mM xanthine; 1 μl methional; 0.1 mM aclacinomycin A or adriamycin, and 13.2 μg of deflavo xanthine oxidase. In some experiments, 40 μg of superoxide dismutase and catalase were added. The reaction was started by the addition of the enzyme and carried out at 37 °C for 30 min.

RESULTS

K_m Values

K_m values of NADPH-cytochrome P-450 reductase for anticancer quinone drugs were determined by measurements of oxygen uptake and oxidation of NADPH.^{3,4)} The results obtained with aclacinomycin A, daunomycin, carbazilquinone and mitomycin C, fitted Michaelis-Menten kinetics and K_m values obtained from Lineweaver-Burk plots are shown in Table I. Carbazilquinone showed the lowest value of 40–44 μM and mitomycin C showed the highest value of 200–227 μM . In the case of adriamycin, the Lineweaver-Burk plot showed substrate inhibition and a slightly concaved curve and thus the K_m value was approximated as 200 μM . Some of these values were lower than the re-

sults reported by Bachur *et al.*,¹²⁾ but were in good agreement with the results reported by Sugioka and Nakano.¹³⁾

Using deflavo xanthine oxidase, the quinone compound dependent xanthine oxidase activity could be assayed only in the presence of a quinone compound by the increase of the absorbance at 292 nm which corresponds to uric acid formation. Without the presence of an anticancer quinone drug, little increase in absorbance at 292 nm occurred. The results obtained with aclacinomycin A, adriamycin, and carbazilquinone fitted Michaelis-Menten kinetics and K_m values obtained from Lineweaver-Burk plots were in the range of 39–53 μM (Table I). The carbazilquinone-dependent oxidase activity was about one half of the anthracycline-dependent activities. In the case of mitomycin C, the xanthine oxidase activity was very low and the K_m value was approximated. Among four drugs, carbazilquinone showed high affinity for both NADPH-cytochrome P-450 reductase and deflavo xanthine oxidase and aclacinomycin A and adriamycin showed high affinity for deflavo xanthine oxidase as did carbazilquinone. Mitomycin C showed the lowest affinity for both enzymes.

Reductive Glycosidic Cleavage Reaction by Deflavo Xanthine Oxidase

Since NADPH-cytochrome P-450 reductase and native xanthine oxidase catalyzed the reductive glycosidic cleavage reaction of anthracyclines under anaerobic conditions,^{1–3)} we attempted to demonstrate the same reaction with deflavo xanthine oxidase. The results are shown in Table II. Using xanthine as an electron donor,

TABLE I. K_m Values of NADPH-Cytochrome P-450 Reductase and Deflavo Xanthine Oxidase for Anticancer Quinone Drugs

Compound	NADPH-cytochrome P-450 reductase		Deflavo xanthine oxidase
	O ₂ Uptake ^{a)}	$\Delta A_{340 \text{ nm}}$ ^{b)}	$\Delta A_{292 \text{ nm}}$ ^{c)}
Aclacinomycin A	161	142	38.5
Adriamycin	200 ^{d)}	200 ^{d)}	43.5
Daunomycin	130	141	—
Carbazilquinone	44	40	52.5
Mitomycin C	227	200	>200 ^{d)}

a), b), and c) Enzyme activities were measured by oxygen uptake, NADPH consumption, and uric acid formation, respectively. d) Approximate value.

TABLE II. Reductive Glycosidic Cleavage Reaction by Deflavo Xanthine Oxidase

Substrate	Electron donor	Product	(nmol/tube)
Aclacinomycin A	Xanthine	7-Deoxyaklavinone	11.1
		7,7'-Bis(7-deoxyaklavinone)	13.5
Aclacinomycin A	Xanthine (Air) ^{a)}	None	0
Aclacinomycin A	NADH	7-Deoxyaklavinone	0
		7,7'-Bis(7-deoxyaklanone)	>0.6
Adriamycin	Xanthine	7-Deoxyadriamycinone	61.2
Adriamycin	Xanthine (Air) ^{a)}	None	0
Adriamycin	NADH	None	0

a) Reaction was carried out under atmosphere of air.

deflavo xanthine oxidase effectively catalyzed the reductive glycosidic cleavage reaction under anaerobic conditions and 7-deoxyaklavinone and its dimer, 7,7'-bis(7-deoxyaklavinone), were produced from aclacinomycin A. Under the same conditions, 7-deoxyadriamycinone was produced from adriamycin. When the reaction was carried out under an atmosphere of air, the reaction did not proceed. These properties were in good agreement with the results obtained from native xanthine oxidase. When NADH was used as an electron donor, little or no reaction occurred with deflavo xanthine oxidase.

ESR Spectrum

Since we had previously demonstrated the importance of the semiquinone intermediate for the action and metabolism of anticancer quinone drugs,³⁻⁷⁾ we, then measured the free radical formation from the quinone drugs in the enzyme systems. The incubation of aclacinomycin A with NADPH-cytochrome P-450 reductase or xanthine oxidase gave free radical signals as shown in Fig. 2 A or B, respectively. The signal at $g = 2.004$ appeared immediately and after 30 min, the signal became weak. With the introduction of air, the signal disappeared. Incubations without the enzyme or anticancer quinone drug produced no signals.

The signals from adriamycin are shown in Figs. 2 C and D. In the NADPH-cytochrome P-450 reductase system, the signal at $g = 2.004$ was symmetric for 5 min, then changed into $g = 2.005$ which is asymmetric (Fig. 2C(a)). In the xanthine oxidase system, symmetric signal at $g = 2.004$ appeared (Fig. 2D(a)). After 15 min, the signal became asymmetric ($g = 2.005$) and intense and was maintained at least for 1 h (Fig. 2D(b)). When air was introduced, the signal dis-

appeared. Although, the hyperfine structure was not observed from aclacinomycin A and adriamycin, the g values and the shape of the signals suggested that the obtained radicals are semiquinone radicals of the drugs.^{12,14-17)}

The signal from carbazilquinone produced by xanthine oxidase is shown in Fig. 2E. The signal at $g = 2.005$ consisted of five peaks, probably originating in the two nitrogens of the aziridinyl groups at positions 2 and 5, and after 10 min, the signal completely disappeared. The hyperfine structures of this and previous studies^{7,18)} indicated that the observed free radical is a semiquinone free radical from carbazilquinone and exists freely in the solution.

In the case of mitomycin C, we did not observe any signals in both enzyme systems.

UV-Visible Spectrum

Under anaerobic conditions, the reductive glycosidic cleavage of anthracyclines and the reductive activation of mitomycin C were catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase.¹⁻⁴⁾ Hence, we measured the changes of UV-visible spectra of the drugs that were produced by these enzymes. In the presence of NADPH-cytochrome P-450 reductase, the absorbance of the peak of aclacinomycin A at 435 nm was slightly decreased in few minutes, then shifted to 450 nm (Fig. 3A). By further incubation, the reduction velocity was lowered and a slight shoulder at 408 nm appeared. The incubation mixture became turbid due to the formation of yellow precipitates. After 3 h and the introduction of air, the shoulder at 408 nm disappeared but the peak at 450 nm remained. The yellow products were identified by TLC as 7-deoxylaklavinone (20%) and its dimer, 7,7'-bis(7-deoxyaklavinone) (80%). The substrate,

FIG. 2. ESR Spectra Obtained from Aclacinomycin A, Adriamycin, and Carbazilquinone in the NADPH-Cytochrome P-450 Reductase and Xanthine Oxidase Systems

A and B were obtained from aclacinomycin A. A: NADPH-cytochrome P-450 reductase system, a; spectrum was recorded after 8 min, b; after the introduction of air. Modulation amplitude was 1 G. B: Xanthine oxidase system, spectrum was recorded after 5 min. C and D were obtained from adriamycin. C: NADPH-cytochrome P-450 reductase system, a; spectrum was recorded after 40 min, b; after the introduction of air. Modulation amplitude was 1 G. D: Xanthine oxidase system, spectra a and b were obtained after 4 and 50 min, respectively. E was obtained from carbazilquinone in the xanthine oxidase system after 4 min. Time constant was 0.1 s.

aclacinomycin A, could not be detected.

When NADPH-cytochrome P-450 reductase was used, the peak of absorbance of adriamycin at 480 nm decreased and a peak at 495 nm appeared (Fig. 3B). Red precipitates ap-

peared after 10 min, and the reaction mixture became turbid. After long incubation, the shoulders at 420, 530, and 575 nm appeared. After 40 min and the introduction of air, the small shoulder at 420 nm disappeared but the peak at 495

nm and the shoulders at 530 and 570 nm remained. The product was identified by TLC and more than 90% of adriamycin was reductively cleaved to 7-deoxyadriamycinone. After a long incubation, a slight increase in absorbance at 408 nm from aclacinomycin A and at 420 nm from adriamycin were observed. Both of these species were assigned as the hydroquinone forms of the products. The same species were obtained by chemical reduction of these drugs using a small amount of solid dithionite in 0.1 M Tris-HCl,

pH 7.5. The reports by Kleyer *et al.*^{19,20)} also showed the formation of these intermediates.

The spectral changes of carbazilquinone, which was produced by NADPH-cytochrome P-450 reductase, are presented in Fig. 3C. The peak absorbance at 326 nm decreased smoothly, and a new peak of low absorbance at 305 nm appeared. After the introduction of air, the original spectrum was obtained slowly, and the recovery of carbazilquinone was confirmed by TLC. The 305 nm species was assigned as the fully reduced

FIG. 3. Spectral Change of Anticancer Quinone Drugs Produced by NADPH-Cytochrome P-450 Reductase

A: Aclacinomycin A, spectra a, b, c, d, and e, were recorded after 0, 2, 15, 91, and 180 min, respectively. B: Adriamycin, spectra a, b, c, d, and e, were recorded after 0, 2, 11, 15, and 30 min, respectively. C: Carbazilquinone, spectra a, b, c, d, and e, were recorded after 0, 1, 4, 7, and 21 min, respectively, and spectrum f was recorded 43 min later after the introduction of air. D: Mitomycin C, spectra a, b, c, d, e, and f, were recorded after 1, 11, 20, 35, 65, and 100 min, respectively.

form of carbazilquinone which was suggested the formation of the same species in a chemical reduction of carbazilquinone with 1 mM sodium borohydride in 0.1 M Tris-HCl, pH 7.5.

In the presence of the reductase, the peak absorbance of mitomycin C at 362 nm decreased with time and new peaks at 244 (not shown) and

310 nm appeared (Fig. 3D). Isosbestic points at 327 and 395 nm were observed. This behavior is similar to that described in a report by Schwartz, where the reduction was carried out with dithionite or with liver homogenate.²¹⁾ After the introduction of air, about 10% of the recovery of the absorbance at 362 nm was calculated and the

FIG. 4. Adrenochrome Formation by Anticancer Quinone Drugs in the NADPH-Cytochrome P-450 Reductase and Deflavo Xanthine Oxidase Systems

A and B were obtained in the NADPH-cytochrome P-450 reductase system. Superoxide dismutase was added at the positions of dashed arrow. A: The results indicated by a, c, d, and g, were obtained from aclinomycin A, and, b and e were obtained from adriamycin. The concentration of aclinomycin A in c was $40 \mu\text{M}$. The results shown by d, e, and g, were obtained in the presence of superoxide dismutase. The result of f was obtained in the quinone drug minus system. B: The results shown by a, c, and e, were obtained from $80 \mu\text{M}$ of carbazilquinone, and, b and d were obtained from mitomycin C. The results indicated by c, d, and e, were obtained in the presence of superoxide dismutase. C and D were obtained in the deflavo xanthine oxidase system. The anticancer quinone drug and superoxide dismutase were added at the positions of solid and dashed arrow, respectively. C: The results shown by a, b, and f, were obtained from aclinomycin A, and c, d, and e, were obtained from adriamycin. The results shown by b, d, e, and f, were obtained in the presence of superoxide dismutase. D: The results indicated by a, b, and e, were obtained from carbazilquinone, and, c and d were obtained from mitomycin C. The results shown by b, d, and e, were obtained in the presence of superoxide dismutase.

rest remained as the 310 nm species without changing the intensity. On TLC, the incubated sample remained at the original position in contrast with authentic mitomycin C (R_f 0.37). Since the 310 nm species showed a peak at 244 nm and a shoulder at 362 nm, the wavelengths of the peaks and their intensities indicated that the 310 nm species is the quinone form of 2,7-diaminomitosene or 2,7-diamino-1-hydroxymitosene or a mixture of both.²²⁻²⁴ Thus, the above results indicate that there was a small amount of the hydroquinone form of mitomycin C in the incubation mixture.

Using the xanthine oxidase system, we carried out similar experiments using xanthine as an electron donor. Upon incubation of aclacinomycin A and adriamycin, the spectra changed similarly as in the case of the reductase system. After a long incubation period, the clear formation of the fully reduced form of aclacinomycin A, a peak at 408 nm, and a small amount of the fully reduced form of adriamycin, a peak at 420 nm, were observed with the concomitant increase in the absorption at 287 nm which corresponded to uric acid formation. Upon introduction of air, the peaks at 408 nm from aclacinomycin A and at 420 nm from adriamycin disappeared and 7-deoxyaglycone metabolites were identified (data not shown). In the case of carbazilquinone and mitomycin C, the spectral changes observed in the region of 320–700 nm were similar to those obtained in the reductase system. The spectra of the fully reduced form of carbazilquinone and the metabolites of mitomycin C were not seen due to the absorption of uric acid (data not shown).

Superoxide Formation

Since we have suggested the formation of superoxide radicals by the reduction of oxygen by the semiquinone form of the quinone drug in enzymes systems under aerobic conditions,⁴⁻⁶ we decided to test the production of superoxide anion radicals by adrenochrome formation from epinephrine. In the NADPH-cytochrome P-450 reductase system, four anticancer quinone drugs effectively stimulated the adrenochrome formation (Fig. 4 A and B). The adrenochrome formation was dependent on the concentration of the quinone drug and, at low concentrations, an initial lag phase was observed. In the presence of superoxide dismutase, the adrenochrome formation was inhibited in a concentration dependent

manner. The system without the quinone drug also produced adrenochrome, but slowly. The efficiency of the adrenochrome formation was in the order of carbazilquinone, aclacinomycin A, adriamycin and finally mitomycin C.

In the case of the deflavo xanthine oxidase system, without the addition of anticancer quinone drugs, only little formation of adrenochrome was observed (Fig. 4 C and D). In the presence of a quinone drug, the adrenochrome formation was stimulated, especially by aclacinomycin A. The order of efficiency was; aclacinomycin A, adriamycin, carbazilquinone, and mitomycin C. The addition of superoxide dismutase inhibited the adrenochrome formation.

FIG. 5. Oxygen Uptake by Anticancer Quinone Drugs in the NADPH-Cytochrome P-450 Reductase and Deflavo Xanthine Oxidase Systems

A and B were obtained in the NADPH-cytochrome P-450 reductase system. A: Carbazilquinone. B: Mitomycin C. The reductase concentration was 7.95 μ g. At the positions of solid and dashed arrow, 0.2 mM anticancer quinone drug and 5 μ g of catalase were added, respectively. C and D were obtained in the deflavo xanthine oxidase system. C: Aclacinomycin A. D: Adriamycin. At the positions of solid and dashed arrow, the anticancer quinone drug and 15 μ g of catalase were added, respectively.

Oxygen Uptake and Production of Hydrogen Peroxide

In a previous report, we demonstrated oxygen uptake and production of hydrogen peroxide, in the presence of aclacinomycin A and adriamycin, in the NADPH-cytochrome P-450 reductase system.³⁾ Similarly, the effects of carbazilquinone and mitomycin C on oxygen consumption and formation of hydrogen peroxide were tested in the reductase system. As shown in Fig. 5 A and B, carbazilquinone and mitomycin C stimulated the oxygen uptake and carbazilquinone was more effective than mitomycin C. After the addition of catalase, oxygen production followed by a low oxygen uptake were observed. At a drug concentration of 0.1 mM, aclacinomycin A, adriamycin and carbazilquinone showed very similar stimulatory effects and mitomycin C was the least effective. The oxygen uptake in the deflavo xanthine oxidase system is shown in Fig. 5 C and D. Before the addition of a quinone drug, little oxygen uptake was observed, and in the presence of aclacinomycin A or adriamycin, oxygen uptake was stimulated. After the addition of catalase, oxygen production followed by low oxygen uptake were observed. In the case of carbazilquinone and mitomycin C, under the same conditions, the oxygen uptake was very low compared to aclacinomycin A and adriamycin. The production of oxygen by the addition of catalase indicated the presence of hydrogen peroxide in the system.

Hydroxyl Radical Generation

Hydroxyl radical is a harmful species in

oxygen toxicity,²⁵⁾ and thus, we measured hydroxyl radical production by determining ethylene formation from methional in the deflavo xanthine oxidase system as in the NADPH-cytochrome P-450 reductase system.^{5,6)} As shown in Table III, ethylene production was observed when aclacinomycin A or adriamycin was added to the deflavo xanthine oxidase system. Adriamycin was more effective than aclacinomycin A. Superoxide dismutase or catalase drastically inhibited the ethylene production. The inhibition by superoxide dismutase differed from the results obtained with the NADPH-cytochrome P-450 reductase system.^{5,6)} The inhibition by superoxide dismutase suggested a role of metal ions in the production of hydroxyl radicals.^{26,27)}

DISCUSSION

For the actions of anticancer quinone drugs to take place, the importance of the semiquinone forms of the drugs and consequently, the reduced oxygen species, has been suggested by many workers. There are many reports that showed the generation of the semiquinone or drug free radicals from aclacinomycin A, adriamycin, daunomycin and carbazilquinone, in the NADPH-cytochrome P-450 reductase,^{7,12,13,15,16)} xanthine oxidase,^{17,22)} microsomal,^{14,18,28)} and chemical reduction systems.^{19,20,29-31)}

In the ESR study, the formation of free radicals from anticancer quinone drugs except from mitomycin C, were evidenced in both the reductase and oxidase systems (Fig. 2). The hy-

TABLE III. *Ethylene Production from Methional by the Presence of Anticancer Quinone Drug in the Deflavo Xanthine Oxidase System*

System	Ethylene produced (pmol/system) (%) ^{a)}	
Assay system	12	—
+Aclacinomycin A	820	100
+Aclacinomycin A +superoxide dismutase	29	3.5
+Aclacinomycin A +catalase	238	29.0
+Aclacinomycin A +superoxide dismutase +catalase	7	0.9
+Adriamycin	1300	100
+Adriamycin +superoxide dismutase	68	5.2
+Adriamycin +catalase	311	23.9
+Adriamycin +superoxide dismutase +catalase	3	0.2

a) Percentage was expressed using aclacinomycin A or adriamycin containing system as 100%.

perfine structure from carbazilquinone indicated the semiquinone form of the drug and its free existence in the solution (Fig. 2E).^{7,18)} Although the hyperfine structure from aclacinomycin A and adriamycin was not observed, the *g* value and the shape of the signal suggested the formation of semiquinone radicals of the drugs (Fig. 2 A, B and D(a)).^{12,14-17)} The signal from alcainomycin A and carbazilquinone disappeared after a long incubation period or after introduction of air, and this behavior was explained by the reduction or oxidation of the semiquinone form of the quinone drugs. In the case of aclacinomycin A, the signal was relatively weak and in the case of adriamycin, the symmetric signal observed progressively changed into a strong and asymmetric one which was maintained for at least 1 h (Fig. 2 C and D). A similar strong and asymmetric signal was reported only by Kalyanaraman *et al.* and Shinha and Gregory in the daunomycin-microsome system.^{14,28)} They suggested that a semiquinone metabolite of deoxyaglycones or anthracycline polymerization products are the major causes for the asymmetric spectrum.

In the UV-visible spectral studies, the fates of the anticancer quinone drugs in the enzyme system were observed (Fig. 3). In the reductase system, about 10% of the absorbance of aclacinomycin A and adriamycin decreased in a few minutes, then gradually changed into 7-deoxyaglycones with the precipitation of these products (Fig. 3 A and B). Further incubation produced a small amount of the fully reduced form of the drugs. In the case of mitomycin C, the compound was reductively activated and converted into mitosene analogues (Fig. 3D). These results were in good agreement with the property of one-electron transfer of NADPH-cytochrome P-450 reductase.³²⁾ On the other hand, the hydroquinone form was produced from carbazilquinone. This was explained by the disproportionate amounts of the semiquinone form of the drug (Fig. 3C).³²⁾ In the case of xanthine oxidase, the formation of the fully reduced form of 7-deoxyaglycone and its dimer was observed after a long incubation period of aclacinomycin A (data not shown). Since xanthine oxidase is a one- and two-electron transfer enzyme, the formation of the fully reduced form may be the result of two-electron reduction.³³⁾ The spectra of the semiquinone form of the quinone drug and more recently reported, 7-

deoxyaglycone tautomer of anthracyclines, were not observed in our system, probably due to both species being transient, and the insufficient amounts, formed under our conditions.^{14,19,20)} For the reductive glycosidic cleavage and reductive alkylation reactions of anthracyclines and the reductive activation of mitomycin C, two mechanisms are proposed. One is the one-electron mechanism which corresponds to the semiquinone intermediate,^{3,22,34)} and the other is the two-electron mechanism which corresponds to the hydroquinone intermediate.^{20,35,36)} Recently, results supporting the two-electron mechanism have been reported in the studies on the reduction of anthracyclines.^{19,20,37)} In the present study, we did not observe preferential formation of the hydroquinone form of anthracyclines and mitomycin C, except for aclacinomycin A in the oxidase system.

In both enzyme systems, the four anticancer quinone drugs tested effectively produced superoxide anion radical and hydrogen peroxide (Figs. 4 and 5). Carbazilquinone and mitomycin C were not as effective as anthracyclines in the deflavo xanthine oxidase system. Hydroxyl radical production in both enzyme systems by anticancer quinone drugs was also observed (Table III).⁵⁾ In contrast to the reductase system,^{5,6)} the hydroxyl radical production in the deflavo oxidase system was dependent on superoxide radicals and this result suggested the reduction of a metal ion by superoxide anion radicals.^{26,27)} It is interesting that adriamycin produced more ethylene than aclacinomycin A.

Using deflavo xanthine oxidase, the electron flow from xanthine to anticancer quinone drugs was clearly evidenced. In the reductive glycosidic cleavage reaction, xanthine was an effective electron donor whereas NADH was ineffective (Table II). These results are in good agreement with the properties of xanthine oxidase which possesses two sites for electron influx, *i.e.* xanthine and NADH sites, and for electron outflow, *i.e.* oxygen and dye binding sites.^{33,38)} Moreover, the anticancer quinone drug can accept an electron from ferrous ion as described in a recent report where anthracyclines accepted an electron from the nonhemeprotein, ferredoxin.³⁷⁾ Xanthine oxidase is a one- and two-electron transfer enzyme, and therefore the semiquinone and hydroquinone forms of the quinone drug would be

FIG. 6. Schematic Illustration of the Interaction of Anticancer Quinone Drug with Xanthine Oxidase and Oxygen

Xanthine Oxidase was indicated by the large oval shaped circle. X, Q, Q $\cdot$, and Q $=$ indicate xanthine, quinone, semiquinone and hydroquinone forms of the anticancer quinone drug, respectively, and Q* indicates the activated and degraded forms of the quinone drug. Solid and dashed arrows indicate the electron flow and the conversion of the molecule, respectively. Open and closed triangles indicate the position of the superoxide dismutase catalysis and inhibition, respectively. Closed box indicate the position of catalase inhibition.

formed. There is, however a disproportionate amount of the semiquinone form of the quinone drug produced.^{32,33)} The dependence on superoxide anion radicals for hydroxyl radical production suggested the Fenton type reaction.^{26,27)} The possible reactions in the xanthine oxidase system are proposed in Fig. 6 and are described as follow:

anaerobic

where, E_{ox} , E_{red} , Q, Q $\cdot$, Q $=$, Me^n , and Me^{n-1} indicate the oxidized and reduced forms of xanthine oxidase, quinone, semiquinone, and hydroquinone forms of the quinone drug, and oxidized and reduced forms of the enzyme metal, respectively.

In summary, using the NADPH-cytochrome P-450 reductase and xanthine oxidase systems, the production of the semiquinone radical of anticancer quinone drugs and the consequent formation of oxygen related radicals and hydrogen peroxide were documented. As reported in previous papers,^{3,39)} enzymes such as DT-diaphorase, mitochondrial NADH dehydrogenase and nuclear NADPH-cytochrome c reductase metabolized anticancer quinone drugs. These enzymes may also interact with the quinone drugs through similar mechanisms. Indeed, the tissue specific toxic effects of anthracyclines were analyzed from the viewpoint of the location of these enzymes in the cells.^{40,41)} Considering the various effects of anticancer quinone drugs, such as alkylation of macromolecules,³⁰⁾ stimulation of lipid peroxidation,^{41,42)} strand scission of DNA,^{15,43,44)} mutagenicity,^{45,46)} and toxicity,^{16,40,41,47)} most of these effects may originate from the drug and oxygen related radicals that are produced by the mechanisms described in this and previous papers.³⁻⁶⁾

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