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Xanthine Oxidase Catalyzed Reductive Cleavage of Anthracycline Antibiotics and Free Radical Formation

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SUMMARY

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Bovine milk xanthine oxidase (xanthine:oxidase oxygen oxidoreductase, EC 1.2.3.2) utilizes anthracycline antibiotics including daunorubicin and adriamycin in its electron transfer system both aerobically and anaerobically and generates drug semiquinone free radicals. Under anaerobic conditions and in the presence of several electron donors, xanthine oxidase metabolizes daunorubicin and adriamycin. The resultant metabolite from daunorubicin was identified as 7-deoxydaunorubicin aglycone by chromatography and mass spectrometry. NADH is the preferred electron donor for the cleavage reaction, although xanthine, purine, and glycolaldehyde also function. The cleavage reaction has the highest activity in phosphate buffer with a wide optimal pH range. With NADH as electron donor the concentration that gave 50% of the maximum cleavage rate is 250 μM for daunorubicin. Oxygen consumption by xanthine oxidase was stimulated by anthracyclines when NADH and NADPH were the electron donors, but it was inhibited if other electron donors were used. Both cleavage reaction and oxygen uptake are inhibited by allopurinol. Daunorubicin semiquinone free radical (g value, 2.0035 ± 0.0001) was detected by electron paramagnetic resonance after a lag period when xanthine oxidase was incubated with NADH or xanthine. The same free radical signal was detected without the lag period when the system was kept anaerobic. This lag period is precisely the time required for the depletion of oxygen as indicated by measuring the oxygen uptake. We propose that the semiquinone free radical form is an intermediate for the reductive glycosidic cleavage of anthracycline.

INTRODUCTION

It is well established that the quinone anticancer drugs, adriamycin and daunorubicin, are metabolized by mammalian microsomes through a reductive glycosidic cleavage, resulting in deoxyaglycone production (1–3). In turn, microsomal pyridine nucleotide utilization, oxygen consumption, and free radical formation are greatly enhanced (4, 5). Further investigations proved that NADPH cytochrome *P*-450 reductase, a flavin-containing microsomal enzyme, catalyzed the reductive glycosidic cleavage (6). Our laboratory and others suggest (7, 8) that the reductive cleavage of anthracyclines by microsomes proceeds first by a single electron transfer to form an anion-free radical intermediate. The free radical then undergoes spontaneous cleavage to yield the deoxyaglycone products. It is our belief that other cellular flavo-enzymes are also capable of catalyzing this reductive cleavage and free radical formation and play a role in the metabolism of the anthracycline antibiotics. Of the flavo-enzymes, xanthine oxidase is well known to use a wide variety of electron donors and to catalyze the reduction of numerous compounds (9). Therefore, we studied the interaction of xanthine oxidase and the anthracycline antibiotics. In this paper, we describe the activity of purified homogeneous bovine milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) as a catalyst for the reductive cleavage of anthracycline antibiotics and for anthracycline conversion to free radical intermediates. A preliminary account of this study has been reported (10).

MATERIALS AND METHODS

Daunorubicin-HCl and adriamycin-HCl were supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, and were further purified as described previously (11). Pyridine nucleotides and flavin coenzymes were purchased from P-L Biochemicals. Bovine milk xanthine oxidase was isolated

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according to the method of Nelson and Handler (12). Deflavo xanthine oxidase was prepared as described by Komai *et al.* (13). Protein was determined by the method of Lowry (14).

Enzymatic activities. Oxygen measurements—The oxygen content of reaction mixtures was measured with a Clark electrode in a Yellow Springs Instrument Model 53 oxygen analyzer. The procedure of Bachur *et al.* (4) was used with some modification. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 1 mM NADH (or other electron donors), 16 to 320 μg of xanthine oxidase as needed and 0.5 mM anthracyclines in a final volume of 1.5 ml.

Anthracycline antibiotic metabolism—The enzyme reaction mixtures were the same as described above for oxygen measurement but were in 0.2 ml total volume. Each reaction mixture was flushed with nitrogen for one minute to produce anoxic conditions. The reaction was started by the addition of enzyme and was carried out at 37° for 10 min with shaking. Anthracycline antibiotics and the metabolic products were extracted into 0.2 ml *n*-butanol, chromatographed and quantified by fluorometric assay as previously described (15).

Spectral analysis. Mass spectra were obtained on a V.G. Micromass 30F spectrometer under the following conditions: ion source temperature was held at 170°, trap current at 170 μA, and electron energy at 70 eV.

Electron paramagnetic resonance (EPR)[1] spectra of anthracycline semiquinones were detected with a Varian E109 spectrometer operated at room temperature, microwave power at 4 mW, and modulation amplitude at 1G. Pitch standard was the reference.

RESULTS

Xanthine Oxidase and Reductive Cleavage of Anthracyclines. The addition of xanthine oxidase to an anaerobic incubation of daunorubicin or other anthracycline antibiotics with electron donors such as xanthine, NADH or NADPH resulted in metabolism of the drugs. When daunorubicin was the substrate, silica gel chromatography of the reaction products showed one major fluorescent metabolite, regardless of the electron donor used. This product had chromatographic characteristics resembling the 7-deoxydaunorubicin aglycone, which is the product of microsomal NADPH cytochrome *P*-450 reductase and is a major metabolite seen *in vivo* (4, 6).

Mass spectrum of aglycone product. The metabolite of daunorubicin from anaerobic cleavage by xanthine oxidase was isolated and analyzed as previously reported (16). Mass spectrum of this compound (Fig. 1) indicated a parent ion peak of *m/e* 382; and fragments at *m/e* 364 ($-H_2O$), 339 ($-COCH_3$), 321 ($-COCH_3$, $-H_2O$). These results together with the thin layer chromatograph clearly indicate that this metabolite is 7-deoxydaunorubicin aglycone and that a reductive cleavage at the C-7 position of daunorubicin occurred. A graphic scheme of the reaction is presented (Fig. 2).

Protein dependence. When NADH was the electron donor, reductive cleavage of daunorubicin responded linearly to the concentration of xanthine oxidase. Boiled xanthine oxidase or incubation without xanthine oxidase yielded no metabolites.

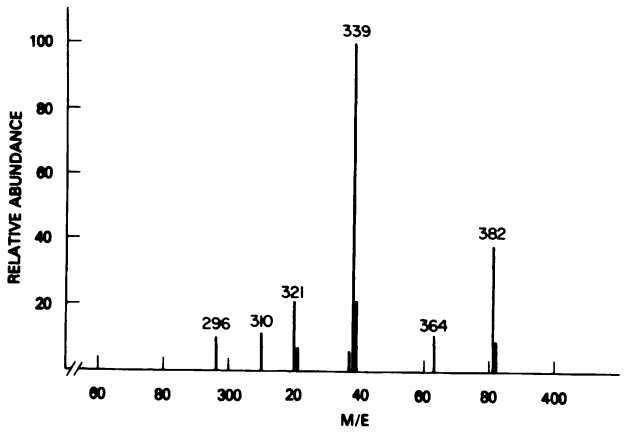

FIG. 1. *Mass spectrogram of the daunorubicin metabolite produced by xanthine oxidase*

The method for isolation and conditions for the spectra are as described in MATERIALS AND METHODS.

pH and ion effects. The cleavage of daunorubicin by xanthine oxidase was determined in three buffers, potassium phosphate, Tris–HCl, and sodium pyrophosphate, all at 0.1 M (Fig. 3). Overall, the enzymatic activity in phosphate buffer was higher than in the other two buffers. Whereas the optimal pH range in phosphate and pyrophosphate seems to be quite wide, in Tris–HCl buffer the optimum was between pH 7.8 to 8.4. All experiments thereafter were performed in 0.1 M phosphate buffer at pH 7.5 unless otherwise noted.

Electron donor specificity of glycosidic cleavage. Since xanthine oxidase utilizes numerous electron donors (9), we examined the abilities of different electron donors to support reductive cleavage of daunorubicin by xanthine oxidase (Table 1). NADH was the most effective electron donor whereas xanthine, glycolaldehyde, and purine yielded lesser amounts of metabolite. When pterine and 1,10-orthophenanthroline were the electron donors, no metabolites were detected, although both were reported to be effective substrates to reduce xanthine oxidase (17). NADPH was active; however, it provided only ½0th of the activity of NADH. Deflavo xanthine oxidase (13) was inactive for glycosidic cleavage when NADH was used as substrate.

Under the optimal conditions used, the rate of daunorubicin cleavage by xanthine oxidase was 0.25 μmol mg^{-1} min^{-1}, which was considerably lower than the cleavage rate by microsomal NADPH cytochrome *P*-450 reductase of 7.12 μmol mg^{-1} min^{-1}.[2] The effect of concentration of daunorubicin on the reductive glycosidic cleavage reaction was determined (Fig. 4). The concentration that gave half of the maximum cleavage rate was 250 μM for daunorubicin. Similar kinetic values were obtained for adriamycin.

Xanthine oxidase catalyzed oxygen consumption. Xanthine is the most efficient electron donor for xanthine oxidase when oxygen is the electron acceptor, as indicated by the highest endogenous specific activity for

[1] The abbreviation used is: EPR, electron paramagnetic resonance.

[2] Unpublished data.

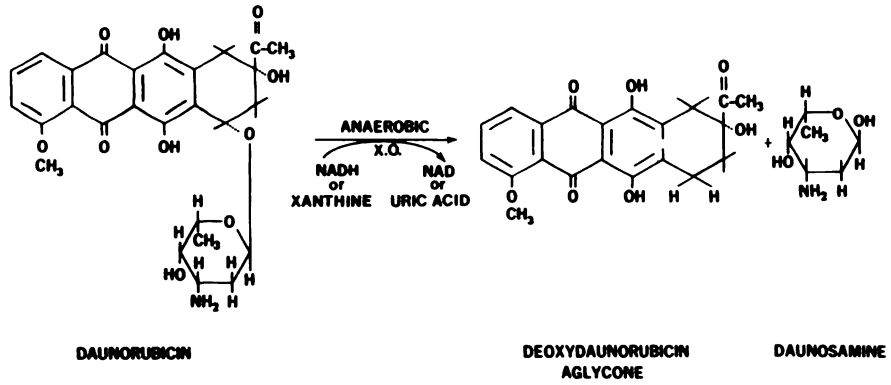

FIG. 2. *Reductive glycosidic cleavage of daunorubicin*
Adriamycin has $—CH_2OH$ for C-14 position instead of $—CH_3$.

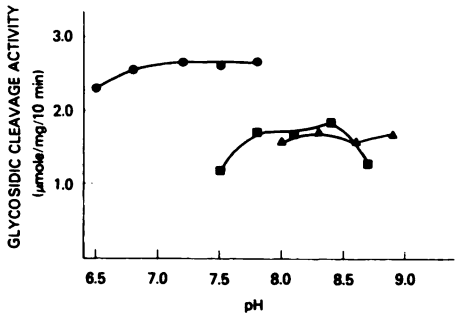

FIG. 3. *pH dependence for glycosidic cleavage of daunorubicin by xanthine oxidase*
The reaction mixture (final volume of 0.2 ml) contained 0.1 M phosphate (pH 7.5), 1.0 mM NADH, 0.5 mM daunorubicin. Each reaction mixture contained 16 μg xanthine oxidase. ●——●, Phosphate buffer; ■——■, Tris-HCl buffer; ▲——▲, pyrophosphate buffer.

TABLE 1
Electron donor specificity for reductive glycosidic cleavage of daunorubicin by xanthine oxidase

Electron donor	Deoxyaglycone produced
(1 mM)	*(μmol/mg/10 min)*
Xanthine	1.14
Glycoladehyde	1.02
Purine	1.52
NADH	2.25
NADPH	0.10
Pterine	0.00
1,10-*ortho*-phenanthroline	0.00

oxygen consumption. This oxygen consumption was inhibited by daunorubicin entirely at 10^{-4} M xanthine (Table 2). At higher concentrations of xanthine (10^{-3} M) the inhibition was only slight. Daunorubicin also inhibited glycolaldehyde and purine supported oxygen consumption to some extent. Of the electron donors we examined, only NADH and NADPH showed increased oxygen utilization when daunorubicin was added to reaction mixtures. The stimulatory action was most profound with NADH, and was studied further. Oxygen consumption, both the endogenous and the anthracyline stimulated, exhibited linear responses to xanthine oxidase concentration. When xanthine oxidase was incubated with NADH (or xanthine) and daunorubicin in a closed system in the presence of air, daunorubicin remained unchanged before oxygen was consumed (Fig. 5). Reductive cleavage of daunorubicin to yield 7-deoxydaunorubicin aglycone started as soon as oxygen was depleted and continued as long as substrates were available. Allopurinol (1 mM), a well described inhibitor of xanthine oxidase, inhibits NADH-dependent daunorubicin cleavage and oxygen uptake about 50%. This inhibition is more dramatic with the xanthine-dependent activities which are inhibited 100%.

Free radical formation. When xanthine oxidase was incubated with daunorubicin in the presence of air and NADH or xanthine as electron donor in an EPR spectrometer, no free radical signal appeared immediately. After a lag period, a strong signal formed. The lag time corresponded precisely to the time required for the depletion of oxygen in the reaction mixture, which was demonstrated by measuring oxygen consumption and EPR signal in the reaction mixture side by side (Fig. 5). The free radical (Fig. 6) had a calculated *g* value of 2.0035 ± 0.0001 when either NADH or xanthine was electron donor. Under anaerobic conditions, the signal appeared without a lag period. Incubation mixtures without xanthine oxidase or daunorubicin under the same conditions gave no signals.

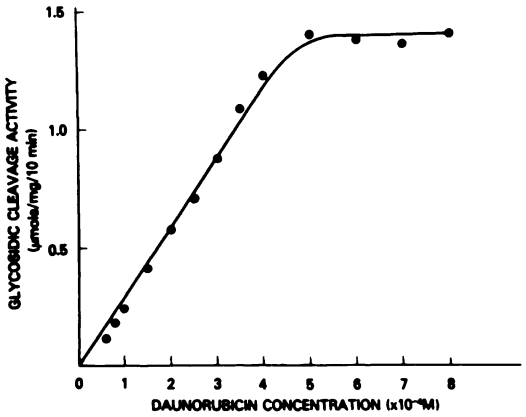

FIG. 4. *Effect of daunorubicin concentration on the glycosidic cleavage catalyzed by xanthine oxidase*
The reactions were carried out as described in Fig. 3. Each reaction mixture contained 12.8 μg xanthine oxidase. The aglycone product was measured as described in MATERIALS AND METHODS.

DISCUSSION

It has been generally accepted that xanthine oxidase receives electrons from xanthine and NADH via different sites (13). The electron flow from xanthine or NADH to acceptor molecules might also occur via different pathways in this enzyme (18). Our data show that anthracycline drugs participate in the electron transfer system of xanthine oxidase; these drugs serve as electron acceptors for the enzyme, receiving electrons from either xanthine or NADH. However, the electron flow from xanthine to anthracycline is less efficient than from NADH to anthracycline. In contrast, when oxygen is the electron acceptor, xanthine is the most effective electron donor. Aerobically, anthracycline facilitated the NADH-to-oxygen electron flow threefold but inhibited the xanthine-to-oxygen flow depending on the concentration of xanthine. We explain these observations as follows: (1) The two reductions, xanthine to oxygen and NADH to oxygen, take two different electron transfer routes. (2) Anthracycline serves as a bridge between NADH and oxygen which facilitates that electron flow pathway; but for the already very effective electron flow of xanthine to oxygen, anthracyclines interfere. (3) Anthracycline as the sole electron acceptor simply is not very effective for the xanthine reduced enzyme. Deflavo xanthine oxidase was unable to cleave anthracycline which indicates the requirement for flavin cofactor for the electron transfer from NADH to anthracycline.

TABLE 2

*Endogenous and daunorubicin affected oxygen consumption of xanthine oxidase**

Electron donor	Concentration	Oxygen uptake		Effect
		Endogenous	Daunorubicin augmented	
	(mM)	*($\times 10^{-7}$ mol/mg/min)*		*(%)*
Xanthine	1.0	16.00	14.10	−12.0
	0.1	22.00	**	−100.0
Glycoaldehyde	1.0	0.87	0.74	−14.9
Purine	1.0	7.30	4.68	−26.0
NADH	1.0	0.91	3.05	225.0
	0.2	0.65	1.75	269.2
NADPH	1.0	0.13	0.21	64.7
	0.2	**	**	
1,10-*ortho*-phenanthroline	1.0	**	**	

* The assays were performed as described under MATERIALS AND METHODS.

** Not measurable.

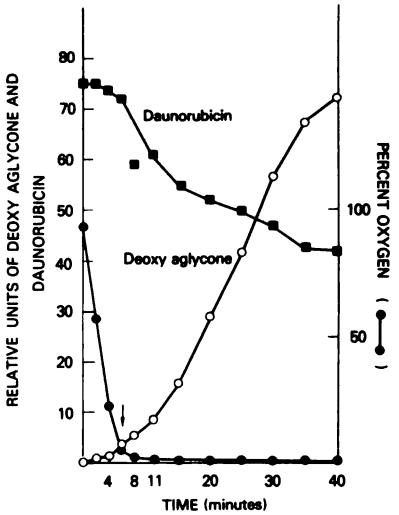

FIG. 5. *Relationship of anthracycline stimulated oxygen consumption to anthracycline biotransformation by xanthine oxidase*

The reaction conditions are as described in MATERIALS AND METHODS. Augmented oxygen uptake began with the addition of daunorubicin (0.5 mM) to the reaction vessel (zero time). 20-μl samples were removed at indicated times and were mixed with 40 μl butanol. A 5-μl portion of the butanol extract was applied to silica gel thin-layer plates and chromatographed in chloroform–methanol–water (80:20:3). The metabolite and drug were detected and assayed as previously described (5). ●——●, Oxygen content; ■——■, daunorubicin; ○——○, daunorubicin deoxyaglycone; beginning of free radical formation (arrow).

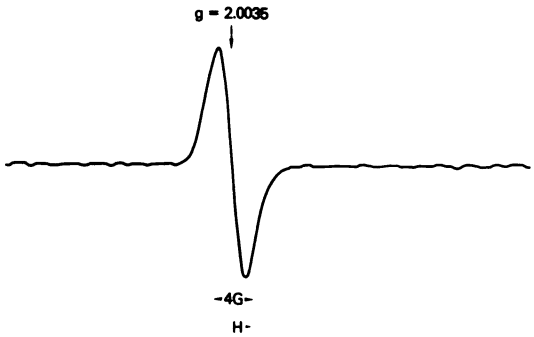

FIG. 6. *EPR spectrum of daunorubicin semiquinone produced by xanthine oxidase*

Reaction mixture and conditions are as described in MATERIALS AND METHODS.

The EPR signal produced from anthracycline reduction by xanthine oxidase (Fig. 6) indicates semiquinone free radical formation of anthracyclines and a single electron transfer mechanism for the reaction. The semiquinone form of anthracycline acts as an intermediate of the electron shuttle, since the free radical signal becomes detectable only when no acceptor is available to remove the electron from the semiquinone. We propose that the semiquinone undergoes spontaneous cleavage to form deoxyaglycone as suggested for the reaction catalyzed by NADPH cytochrome *P*-450 reductase (7, 8). This would also explain the immediate anaerobic cleavage of anthracycline antibiotics where semiquinone signal is seen without any lag period and the end product was also 7-deoxyaglycone. Although the *g* value of 2.0035 is higher than our previous report of 2.0023 (4), we felt that different measuring systems may have contributed to the difference. Our most recent studies agree with the value 2.0035.

The capability of xanthine oxidase to utilize anthracycline antibiotics in its electron transfer system and generate free radicals may play an important role in terms of drug toxicity. Anthracyclines are converted to free radicals which may react directly with susceptible molecules such as DNA, or indirectly generate other toxic free radicals such as $O_2^{\cdot-}$ or $OH^{\cdot}$. Although the efficiency of xanthine oxidase for the conversion may not be as high as microsomal NADPH cytochrome *P*-450 reductase (anaerobic cleavage by xanthine oxidase is only

1/30th that of NADPH cytochrome *P*-450 reductase), the wide distribution and the high content of this enzyme in cells increases its importance.

ACKNOWLEDGMENTS

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REFERENCES

1. Asbell, M. A., E. Schwartzbach, F. J. Bullock and D. W. Yesair. Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. *J. Pharmacol. Exp. Ther.* **182:** 63–69 (1972).
2. Bachur, N. R. and M. V. Gee. Oxygen sensitive reductive cleavage of daunorubicin and adriamycin. *Fed. Proc.* **31:** 835 (1972).
3. Bachur, N. R. and M. V. Gee. Microsomal reductive glycosidase. *J. Pharmacol. Exp. Ther.* **197:** 681–686 (1976).
4. Bachur, N. R., S. L. Gordon and M. V. Gee. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Mol. Pharmacol.* **13:** 901–910 (1977).
5. Bachur, N. R., S. Gordon and M. V. Gee. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.* **38:** 1745–1750 (1978).
6. Oki, T., T. Komiyama, H. Tone and T. Inui. Reductive cleavage of anthracycline glycosides by microsomal NADPH-cytochrome c reductase. *J. Antibiot.* **30:** 613–615 (1977).
7. Bachur, N. R. Anthracycline antibiotic pharmacology and metabolism. *Cancer Treat. Rep.* **63:** 817–820 (1979).
8. Mason, R. P. Free radical metabolites of foreign compounds and their toxicological significance, in *Review of Biochemical Toxicology* (Hodgson, Bend, and Philpot, eds.). Elsevier, New York, 151–200 (1979).
9. Massey, V. Iron–sulfur flavoprotein hydroxylases, in *Iron–Sulfur Proteins* (W. Covenberg, ed.). Academic Press, New York, 301–360 (1973).
10. Pan, S. and N. R. Bachur. Xanthine oxidase catalyzed reductive glycosidic cleavage and free radical formation of anthracycline antibiotics. *Fed. Proc.* **38:** 663 (1979).
11. Bachur, N. R. and J. C. Cradock. Daunomycin metabolism in rat tissue slices. *J. Pharmacol. Exp. Ther.* **175:** 331–337 (1970).
12. Nelson, C. A. and P. Handler. Preparation of bovine xanthine oxidase and the subunit structures of some iron flavoproteins. *J. Biol. Chem.* **243:** 5368–5376 (1968).
13. Komai, H., V. Massey and G. Palmer. The preparation and properties of deflavo xanthine oxidase. *J. Biol. Chem.* **244:** 1692–1700 (1969).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275 (1951).
15. Bachur, N. R. and M. V. Gee. Daunorubicin metabolism by rat tissue preparations. *J. Pharmacol. Exp. Ther.* **177:** 567–572 (1971).
16. Bachur, N. R. Daunorubicinol, a major metabolite of daunorubicin: Isolation from human urine and enzymatic reactions. *J. Pharmacol. Exp. Ther.* **177:** 573–578 (1971).
17. Massey, V. and G. Palmer. On the existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones. *Biochemistry* **5:** 3181–3189 (1966).
18. Nahamura, M. and I. Yamazaki. One-electron transfer reaction in biochemical systems VII. Two types of electron outlets in milk xanthine oxidase. *Biochim. Biophys. Acta* **327:** 247–256 (1973).

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