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ANTHRACYCLINE-INDUCED OXYGEN CONSUMPTION AND OXIDATIVE DAMAGE IN RAT LIVER MICROSOMES ARE NOT NECESSARILY COUPLED

A study with 8 structurally related anthracyclines

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The stimulative effect of 8 anthracyclines (the parent compounds daunorubicin and doxorubicin and 6 structurally closely related anthracyclines) on the production of thiobarbituric acid (TBA)-reactive material was investigated in liver microsomes. Except for daunorubicinone and doxorubicinone, all derivatives stimulated NADPH-dependent production of TBA-reactive material. Doxorubicinone had no effect, daunorubicinone inhibited TBA-reactivity at concentrations up to 50 μM. However, the latter two compounds stimulated oxygen consumption in the presence of EDTA to a degree comparable to that induced by the parent compounds. Since the oxygen uptake under these circumstances represents redox cycling of the drugs, apparently redox cycling and production of TBA-reactive material were not coupled for these compounds.

Spectral measurements showed no decisive role for interaction with free iron (Fe^{3+}) ions in the non-coupling of redox cycling and production of TBA reactive material. Evidence for a role of bound iron ions was not obtained.

It is discussed that for the aglycones oxygen consumption and production of TBA reactive material might be non-coupled through their different interaction with microsomal RNA.

Key words: anthracyclines; oxygen consumption; oxidative damage; microsomes; difference spectra

INTRODUCTION

The drugs adriamycin (doxorubicin) and daunorubicin are effective antineoplastic agents towards a variety of malignancies. A wide use of these drugs is however limited by a maximal allowable cumulative dose (for adriamycin 550 mg/m^2) that has been recommended to confine the incidence of delayed cardiomyopathy (rev. 1). This restriction has lead investigators to synthesize structurally related anthracyclines.

Some of them, such as 4′-epidoxorubicin and 4-demethoxydaunorubicin, have entered the phase of clinical study.

It has been suggested[2] that induction of free radical formation and the attack of cell components by these radicals are underlying the cardiomyopathy. In *in vitro* studies it has been demonstrated that enzymically one-electron reduction of adriamycin or daunomycin results in the formation of a semiquinone (Figure 1, route a). Subsequent reoxidation leads to the original compound and to transfer of the free electron to oxygen (Figure 1, step b). By passing several times through the cycle of reduction and reoxidation, one drug molecule can activate many oxygen molecules[3,4,5].

In liver microsomes the cycle is induced mainly by NADPH-cytochrome P-450 reductase; an increase in oxygen consumption (through route b) and oxidative damage of the membrane (via step c) occur on incubation of microsomes with adriamycin[6,7].

In this study we compare these phenomena for 8 narrow related anthracyclines, the parent compounds adriamycin ③ and daunorubicin ① and 6 derivatives, most of which have been developed as alternatives for the parent compounds. These derivatives are: daunorubicinone ②, doxorubicinone ④, 4-demethoxydaunorubicin ⑤, 4′-O-methyldoxorubicin ⑥, 4′-deoxydoxorubicin ⑦ and 4′-epidoxorubicin ⑧ (Figure 2).

An observed divergence between redox cycling and stimulation of membrane damage for two of the compounds suggested that step c in figure 1 was not similar for all derivatives. The idea that interaction with membrane bound metal ions (such as in cytochrome P-450) could be involved prompted us to give closer attention to the interaction of the different anthracyclines with the microsomal membrane.

MATERIALS AND METHODS

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Bakers Yeast), ribonucleic acid (RNA) (calf liver type IV), flavin mononucleotide (FMN), flavine adenine dinucleotide (FAD), cytochrome c (horse heart type III), $NADP^+$ and NADPH were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Catalase (from beef liver) was from Boehringer Mannheim GmbH, F.R.G. Daunorbicin.HCl ①, daunorubicinone ②, doxorubicin.HCl ③, doxorubicinone ④, 4-demethoxydaunorubicin.HCl ⑤, 4′-O-methyldoxorubicin.HCl ⑥, 4′-deoxydoxorubicin.HCl ⑦ and 4′-epidoxorubicin.HCl ⑧ were a gift from dr. F. Arcamone, Farmitalia Carlo Erba, Milan, Italy. All other chemicals used were of analytical grade purity.

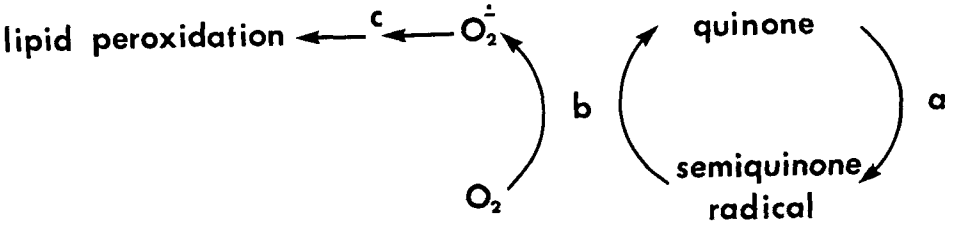
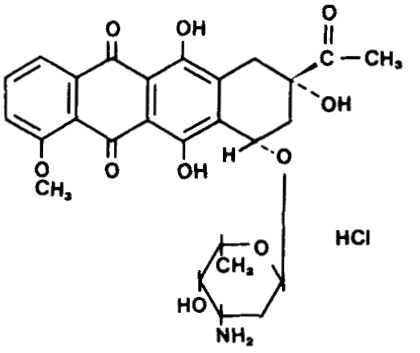
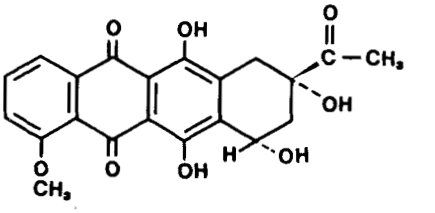


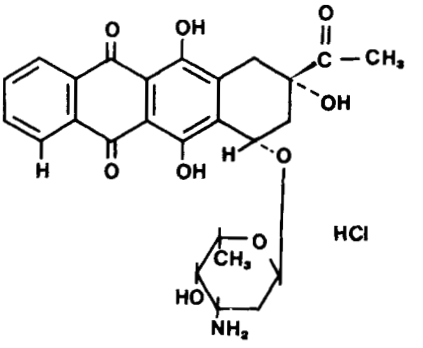
FIGURE 1 Proposed reaction pathway for adriamycin (ADM) in microsomes.



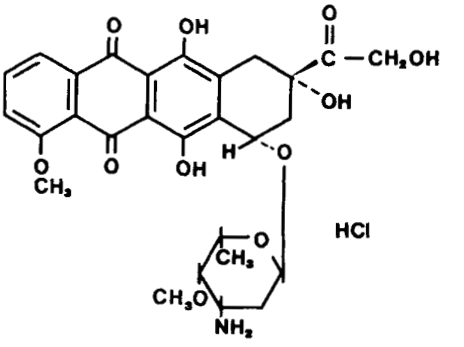
1: daunorubicin.HCl



2: daunorubicinone



5: 4- demethoxydaunorubicin.HCl

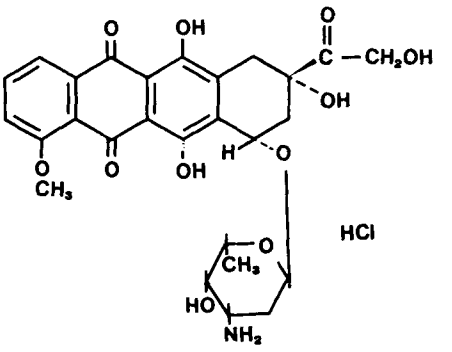


6: 4′-O-methyldoxorubicin.HCl

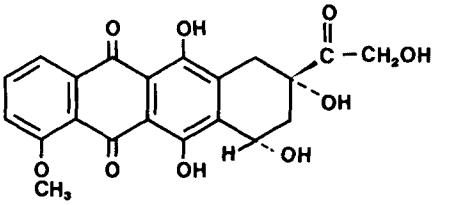
FIGURE 2 Molecular structure of the parent compounds daunorubicin ① and doxorubicin ③ and their derivatives used in this study.

Preparation of Microsomes

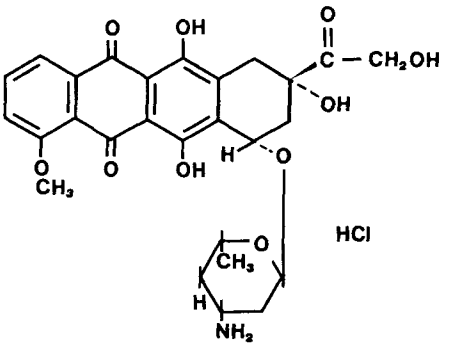
Male Wistar rats (TNO, Zeist, The Netherlands) of 200–250 g weight were used. For the isolation of microsomes 3 rats were decapitated and the livers were removed and homogenized (1:2 w/v) in ice-cold 50 mM phosphate buffer pH 7.4 plus 0.10 mM EDTA. Microsomes were isolated by centrifugation as described[8]. The microsomal pellet was resuspended in phosphate buffer containing 0.10 mM EDTA and stored at −80°C until use.



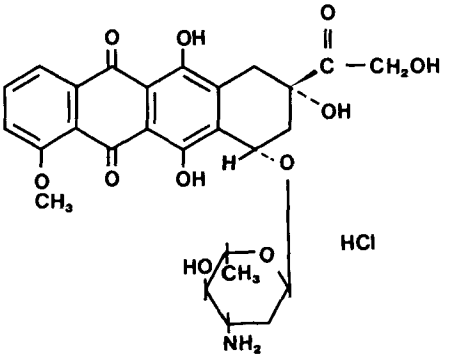
3: doxorubicin.HCl



4: doxorubicinone



7: 4′-deoxydoxorubicin.HCl



8: 4′-epidoxorubicin.HCl

FIGURE 2

Incubation Conditions for the Induction of Membrane Damage

To remove EDTA the microsomes were thawed, diluted and washed with ice-cold 50 mM Tris-HCl, 150 mM KCl pH 7.4 as described previously[8]. The microsomes were used in a final concentration of 1.3 mg protein/ml. Membrane damage was induced by a NADPH-regenerating system[8] as described by Mimnaugh et al.[6]. Anthracyclines were added in DMSO† the final DMSO concentration was 0.5% v/v. The incubation was started with the addition of glucose-6-phosphate dehydrogenase (0.85 U/ml incubate) and was performed at 37°C in a shaking water bath; air was freely admitted.

Microsomal oxidative damage was assayed as thiobarbituric acid-reactive

† DMSO: duriethylsulphoxide.

material[9]. Zero time samples were taken to correct for the extinction of the anthracyclines. The oxidative damage is expressed as nmol malondialdehyde (MDA) per mg protein, using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 533 nm.

Oxygen Consumption

Oxygen consumption was measured at 30°C in an oxygraph fitted with a Clark-type electrode, essentially as described[10]. The reaction volume was 1.4 ml; the buffer was 50 mM Tris-HCl pH 7.5 containing 0.10 mM EDTA and 0.25 mM sucrose. The final concentrations were: 1.0 mg protein/ml, 0.5 mM NADPH, and when indicated 2 mM $NADP^+$. The anthracyclines were used at final concentrations of 100 μM, except for daunorubicinone and doxorubicinone (50 μM). The anthracyclines were added in DMSO (final concentration 0.5% v/v).

Difference Spectra of the Anthracyclines

Difference-binding spectra of the anthracyclines to microsomes were recorded with a double beam Aminco spectrophotometer (DW-2a, UV-Vis), using so-called tandem cuvettes. The sample cuvette contained in one chamber the microsomes (in phosphate buffer) plus the anthracycline (in buffer with DMSO); in the other chamber buffer plus DMSO. The reference cuvette contained in the one chamber the anthracyclines in buffer plus DMSO, in the other microsomal protein plus DMSO (see Figure 3). Baselines were produced by omittance of the anthracyclines, Spectra were recorded from 350–550 nm.

The concentration of microsomal protein was 2 mg/ml; anthracyclines were used in a concentration of 40 μM. The final concentration of DMSO was 10% v/v. This high DMSO concentration was used to prevent precipitation of the aglycones daunorubicinone and doxorubicinone in the absence of microsomal protein. With other anthracyclines it was found that this DMSO concentration did not influence the difference spectra.

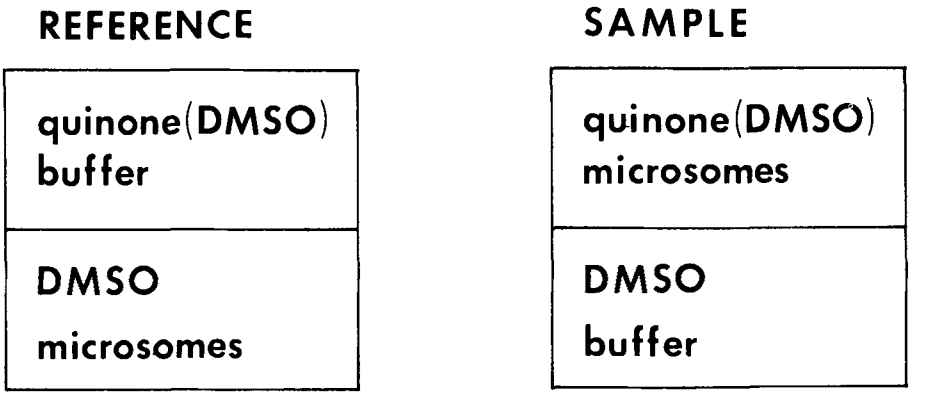


FIGURE 3 Experimental setting for the measurement of the absorbance difference spectra of the anthracyclines (see Materials and Methods).

The difference binding spectra in the presence of RNA were measured in the same way, except that RNA was used instead of microsomes. RNA was used at a concentration of 1 μg/ml.

For the recording of the binding difference spectra induced by iron ions, all solutions were made in demineralized water. Fe^{3+}-ions were used in a final concentration of 3 μM. In this case the final DMSO concentration was 5% v/v.

Difference spectra in the presence of FMN, FAD, catalase and cytochrome c were obtained essentially as described for microsomal protein. FAD and FMN were used in a concentration of 10 μM, catalase and cytochrome c in a concentration of 0.25 mg/ml.

Protein was measured according to Lowry *et al.*[11], using bovine serum albumin as a standard.

RESULTS

Figure 4 shows the NADPH-dependent formation of peroxidative products in liver microsomes induced by the different anthracyclines. The production of thiobarbituric acid-reactive material was measured at three concentrations of the anthracyclines (20, 50 and 100 μM) except for compounds ② (daunorubicinone) and ④ (doxorubicinone) which were insoluble at the higher concentration. Most anthracyclines stimulated the microsomal oxidative damage. Compound ④, however, had no significant effect. Compound ② even inhibited the basic production of TBA-reactive material. For compound ⑤ (4-demethoxydaunorubicin) the stimulation found at the lower concentration decreased with increasing concentration.

Figure 5 shows the effect of the anthracyclines on the NADPH-dependent oxygen consumption in microsomes. Compounds ② and ④ were used at 50 μM, other anthracyclines were used at 100 μM..All drugs significantly stimulated the basic oxygen consumption. $NADP^+$, which inhibits NADPH-cytochrome P-450 reductase[10], decreased the basic oxygen uptake. $NADP^+$ also reduced the anthracycline-induced enhancement of oxygen consumption, except if compound ⑤ was used. This drug was the most powerful stimulator of oxygen consumption in the absence of $NADP^+$.

Figures 4 and 5 show that the drug-induced enhancement of oxygen consumption did not always lead to enhanced production of TBA-reactive material (see compounds ② and ④).

Iron ions, either free or membrane bound, are likely to be involved in the stimulation of oxidative damage[12,13,14,15]. It is known that iron ions interact with anthracyclines, changing their visible spectrum[16]. In the membrane, iron ions in for example cytochrome P-450 could be involved. Therefore, we first looked at the interaction of the anthracycline with the microsomes, by way of spectral measurements.

A difference spectrum was recorded, using the set-up given in Figure 3. Figure 6a shows the resulting difference spectra obtained with liver microsomes. Compounds ①, ③, ⑤, ⑥, ⑦ and ⑧ gave a difference spectrum with a clear minimum in the region of their λ_{max} (for compounds ①, ③, ⑥, ⑦ and ⑧ λ_{max} was 475 nm with a shoulder at 490 nm and for compound ⑤ λ_{max} was 478 nm with a shoulder at 458 nm). Compounds ② and ④ showed no such a minimum. Heart microsomes, which contain only minimal amounts of cytochrome P-450, gave a similar spectrum as liver microsomes. The ΔE at the minimum was even greater than with liver microsomes at the same protein concentration (data not shown). Other heme-containing proteins

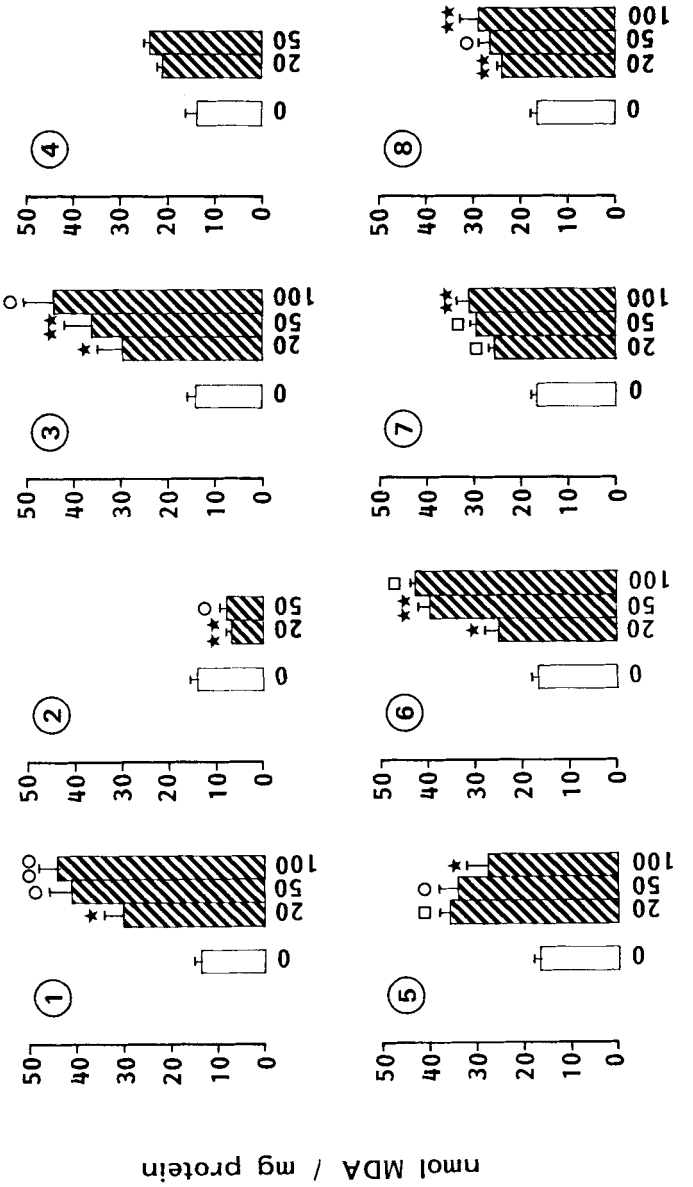


FIGURE 4 Production of thiobarbituric acid-reactive material in liver microsomes in the absence or presence of anthracyclines. Thiobarbituric acid reactive-material is expressed as nmol malondialdehyde (MDA) per mg protein. TBA-reactivity was measured after 90 min of incubation. For other conditions see Materials and Methods. The numbers at the absis refer to the concentration of the anthracyclines ①–⑧ in the incubate.

□ no anthracycline present
▨ anthracycline present

Significancies were calculated using the Students T-test, for the MDA production in the presence of the anthracycline versus the MDA production in the absence of anthracycline. N = 4.

* $p < 0.05$; ** $p < 0.02$; o $p < 0.01$; oo $p < 0.0002$; □ $p < 0.001$.

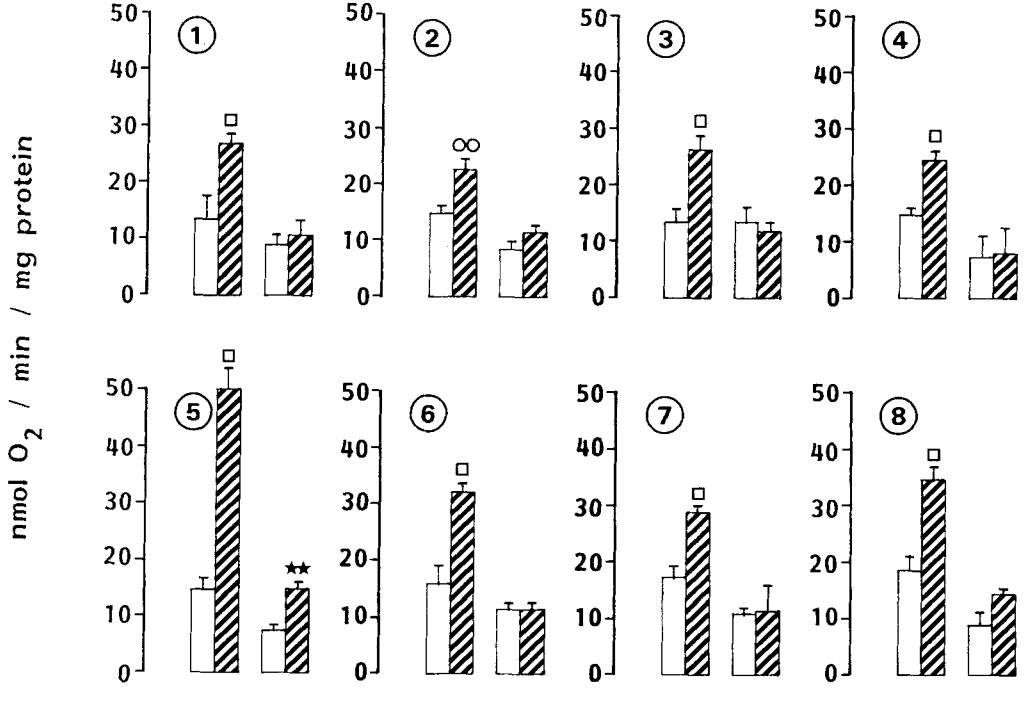


FIGURE 5 Oxygen consumption by liver microsomes and stimulation by anthracyclines ①-⑧.
Oxygen uptake was measured as described in materials and methods, in the absence or presence of the anthracyclines. The concentration of the drugs was 100 μM for compounds ①, ③, ⑤, ⑥, ⑦ and ⑧ and 50 μM for compounds ② and ④. Oxygen consumption was followed for about 5 min after addition of NADPH, and then for another 5 min after addition of the anthracycline.
Left two bars represent the consumption of oxygen in the absence of inhibitor, the right two bars the oxygen consumption in the presence of 2 mM $NADP^+$.

□ O_2 uptake in the absence of anthracycline
▨ O_2 uptake in the presence of anthracycline

The significance of the stimulation of the oxygen consumption was calculated using the Students T-test. N = 4. Symbols for significance as in figure 4.

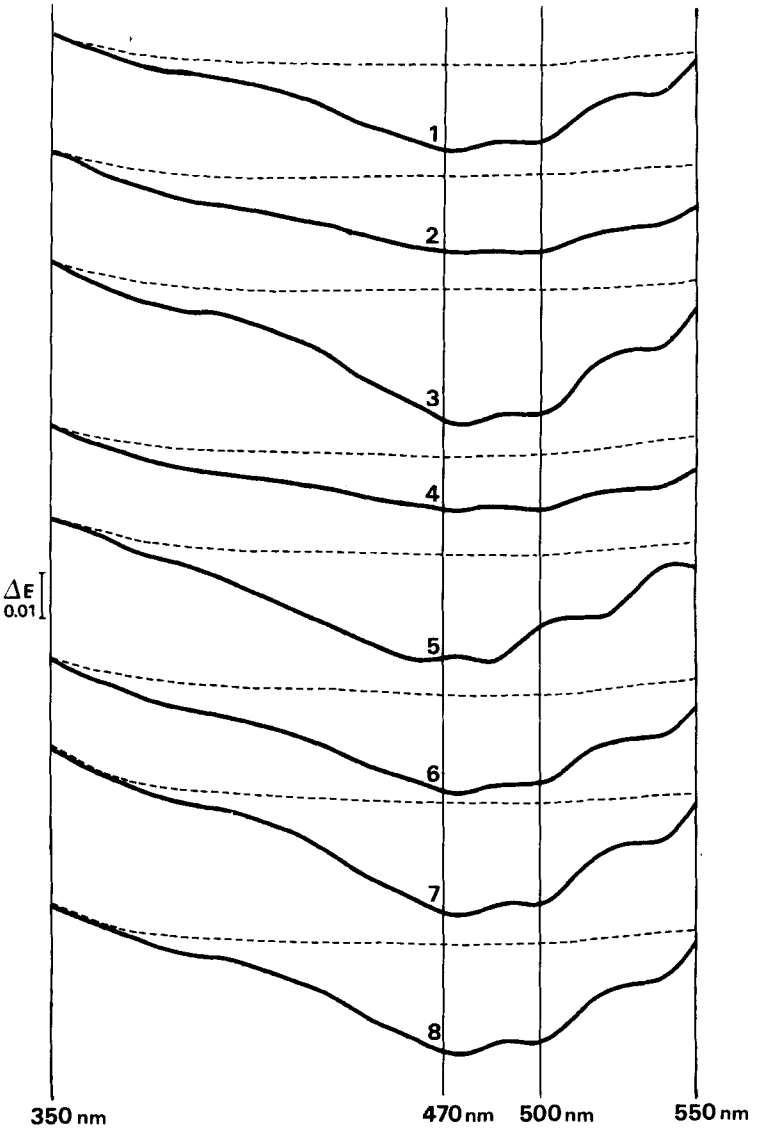


FIGURE 6 Difference spectra recorded for anthracyclines ①-⑧.
The concentration anthracycline was 40 μM. For experimental details see materials and methods and fig. 3.

6.a. difference spectra in the presence of 2 mg liver microsomal protein/ml

which we tried (catalase and cytochrome c) gave no difference spectra with the anthracyclines.

Interaction with free iron ions was then studied (Figure 6b). The concentration of Fe^{3+} was in the range of that which might have been present as contaminant in the buffers in which the peroxidation experiments were performed ($\pm$ 1 μM). It induced for one of the parent compounds (daunorubicin at 40 μM) a maximal ΔE comparable to that obtained with 2 mg liver microsomal protein/ml. No resemblance was found to the microsome-induced spectra. For example, compounds ② and ④ showed a strong interaction (a large ΔE) with Fe^{3+} at 475 nm and not with microsomal protein. Others such as compounds ③ and ⑥ (4′-O-methyldoxorubicin) gave a large ΔE around 475 nm with microsomal protein, but not with Fe^{3+}. Also no correlation was found between the effect on the production of TBA-reactive material and the spectral changes with free Fe^{3+}-ions (see for example compounds ② and ⑦, or compounds ① and ⑥).

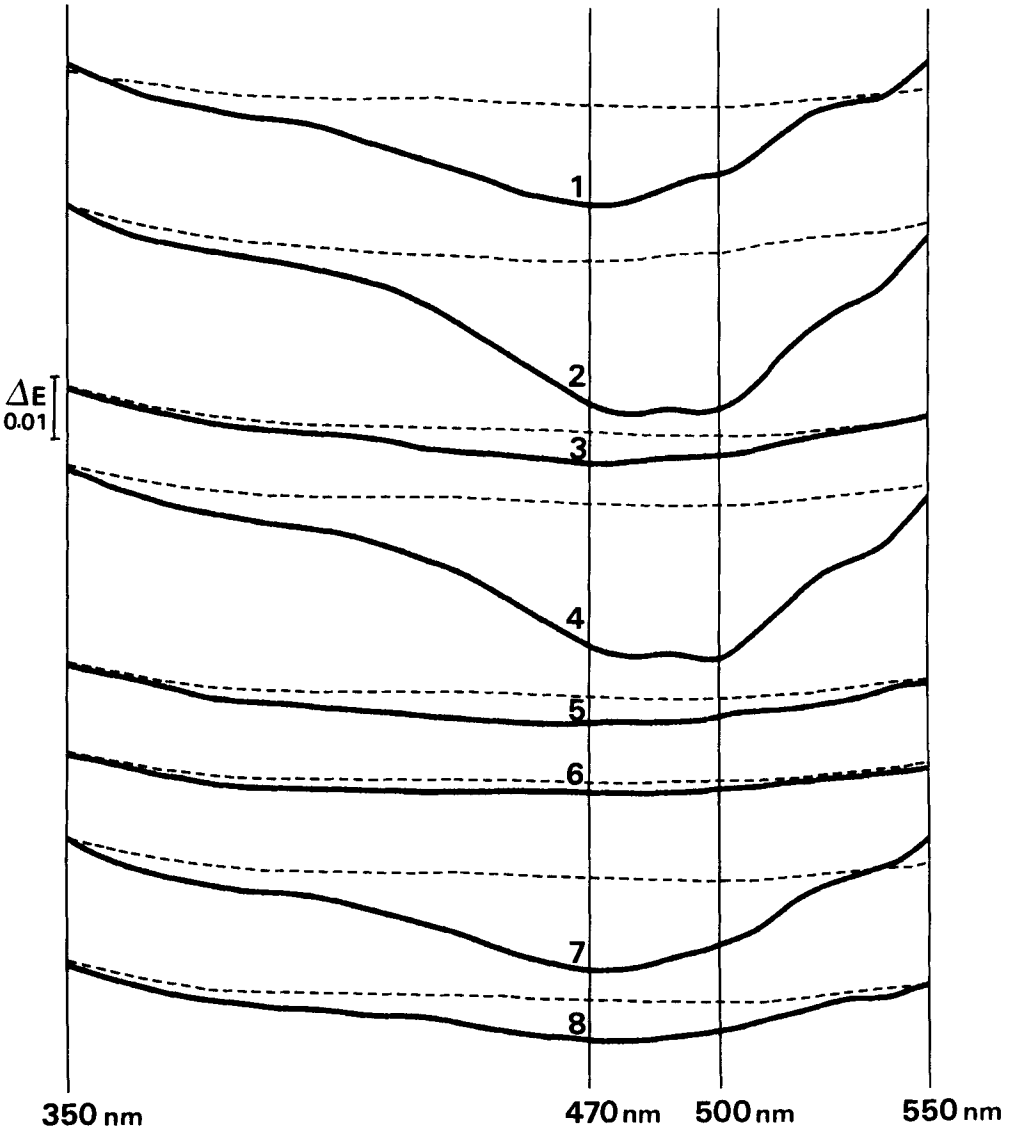
We further tried to clarify the origin of the microsomal interaction with the anthracyclines. Of the compounds known to interact with anthracyclines we tested FAD, FMN and RNA. FAD and FMN, at a concentration of 10 mM, caused no changes in the absorption spectra of the anthracyclines. The spectra obtained with RNA (Figure 6c) more resemble those obtained in the presence of microsomal protein than the Fe^{3+}-induced difference spectra.

DISCUSSION

The compounds 4′-O-methyldoxorubicin ⑥, 4′-deoxydoxorubicin ⑦ and 4′-epidoxorubicin ⑧, modified in the amino-sugar group, are potent stimulators of NADPH-dependent production of TBA-reactive material. At zero drug concentration the stimulating effect was significantly different from that by adriamycin. Compound ⑤ (4-demethoxydaunorubicin) was found to be more effective as the concentration decreased. A decrease in stimulating effect found at higher concentrations has been described also for other anthracyclines[17]. The aglycones, daunorubicinone ④ and doxorubicinone ② were bad inducers of TBA-reactivity. Doxorubicinone led to a small but insignificant increase, daunorubicinone significantly decreased production of TBA-reactive material.

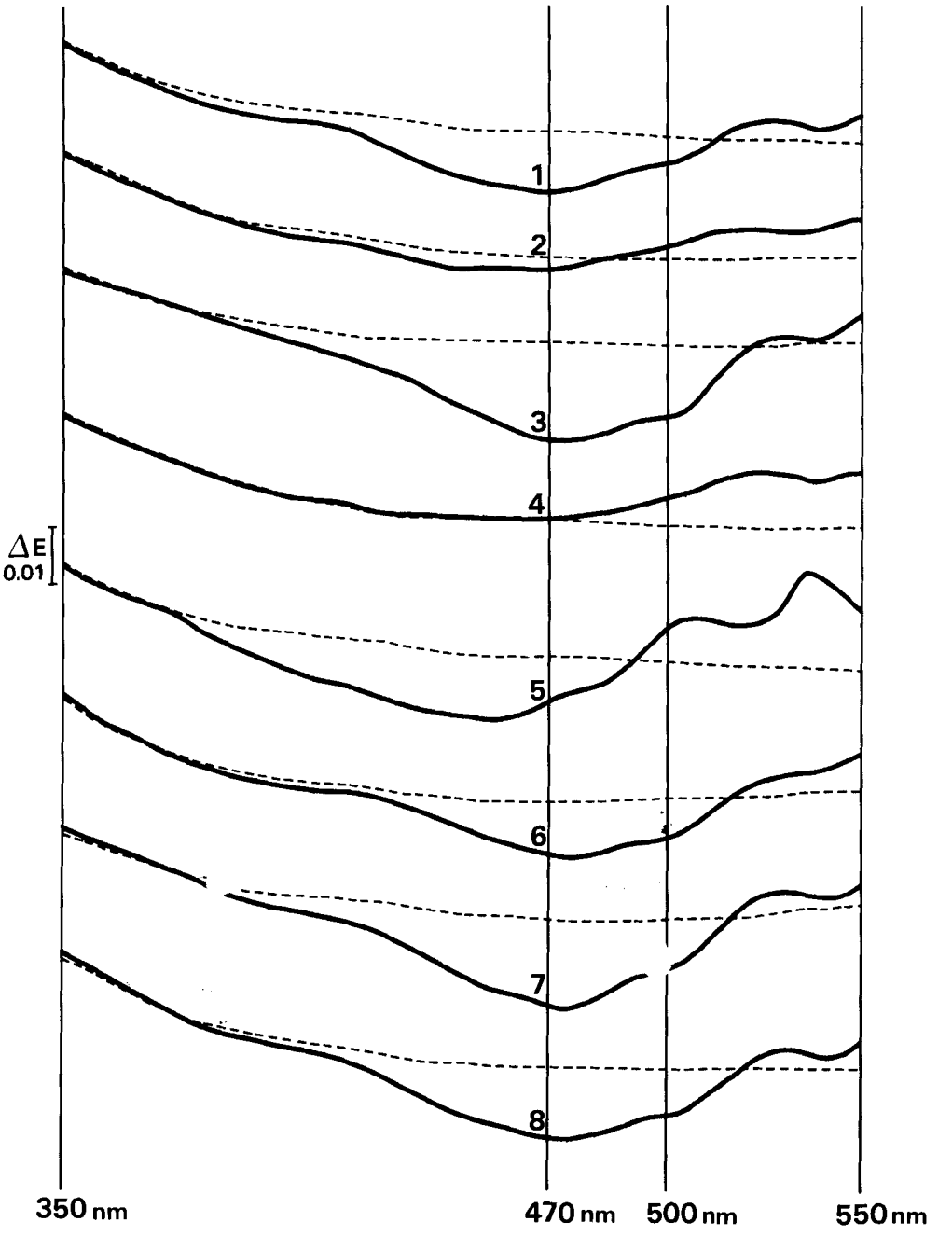
Redox cycling of the anthracyclines as shown in Figure 1 (steps a and b) is important in the stimulation of lipid peroxidation. Since the lack of stimulation of TBA-reactivity of the aglycones was probably due to a lack in activation, oxygen consumption was measured. In the presence of EDTA, when lipid peroxidation was prevented, oxygen uptake is expected to be due mainly to redox cycling and not to incorporation into membrane lipids. From the fact that $NADP^+$ inhibited the stimulation of oxygen consumption induced by the anthracyclines it may be derived that the anthracyclines were all activated by NADPH-cytochrome P-450 reductase. The stimulation of oxygen uptake in the absence of inhibitor demonstrates redox cycling for all anthracyclines. The fact that the aglycones did not enhance the production of TBA-reactive material but underwent redox cycling demonstrates that these two processes are not always tightly coupled. In this study, using constant conditions, the type of drug was found to determine the coupling. The coupling between drug activation and lipid peroxidation might also depend on the conditions. For adriamycin this has been demonstrated for example by Sugioka *et al.*[12,18].

Metal ions play an important role in the coupling of activation and lipid peroxida-



6.b. idem in the presence of 3 μM Fe^{3+}

tion[12,13,14,15]. In the absence of iron ions or ions of other metal salts lipid peroxidation is slow because of lack of good initiators. The superoxide anion radical (shown in Figure 1) is a bad initiator of lipid peroxidation. In the presence of metal catalyzers other reactive oxygen species are formed ($OH^{\cdot}$ radicals, perferryl ion) which might more easily attack unsaturated fatty acids. Also it is evident now, that in the presence of metal ions adriamycin can induce damage to DNA or lipids without activation to a semiquinone[19,20,21].



6.c. idem in the presence of 1 μg RNA/ml

If metal (iron) ions were involved in differences between the stimulative effects of the anthracyclines ①-⑧ these ions should be either membrane bound or free. Membrane bound iron is found in cytochrome P-450, an important component of liver microsomal membranes. The difference spectra obtained on addition of the anthracyclines to liver microsomes showed interaction with membrane components. Yet, not P-450 heme caused this interaction since with heart microsomes, having insignificant amounts of P-450, even larger absorbance differences were found. Other iron containing proteins, such as catalase and cytochrome c, gave no difference spectrum when added to the anthracyclines.

The difference spectra with free iron (Fe^{3+}) ions demonstrate that binding of free iron *per se* cannot cause the differences found in stimulation of TBA-reactivity of the anthracyclines. The aglycones, being bad stimulators of TBA-reactivity, appeared to be excellent complex-formers with iron. At the other hand drugs that stimulated lipid peroxidation were both found in the category of compounds that showed little interaction with iron (③, ⑤, ⑥, ⑧) and in the group that clearly showed interaction (①, ②, ④, ⑦).

Of other substances tested (FAD and FMN at 10 μM, RNA at 1 μg/ml) only RNA affected the anthracycline absorption. The difference spectra obtained in this case resembled those obtained with microsomes. The aglycones, lacking the amino-sugar group, gave less sharp difference spectra than the other anthracyclines. Indeed, it is known[16] that the amino-group in the sugar-moiety is necessary for intercalation with DNA and RNA.

Summarized, these results show that activation of anthracyclines and transfer of the free electron to oxygen does not necessarily lead to measurable stimulation of TBA-reactivity. It is not clear which factor(s) determine a coupling of these two events. Complex formation of the quinones with iron ions (either membrane bound or free) does not seem to be decisive.

Fixation of the anthracycline to the membrane might be a factor that determines the stimulation of TBA-reactivity in the microsomal membrane. We found that the aglycones which showed little interaction with the microsomal membrane were also bad inducers of TBA-reactivity. Similarities in spectral changes suggested that the interaction of the anthracyclines with the microsomal membrane was via microsomal RNA. RNA is present in the microsomal preparations at a concentration of about 5 mg/100 mg protein. Blocking the amino-group which is involved in the interaction with RNA should then lead to reduced membrane damage, even if redox cycling occurs. AD-32, an adriamycin derivative in which the amino-group is blocked by a trifluoroacetyl-group, is an interesting compound in this respect.

Our results also point to the fact that the reduced cardiotoxicity reported for some new anthracyclines is certainly not due to a less powerful stimulation of free radical formation and of membrane damage. For the compounds given in table I other factors (e.g. pharmacokinetic parameters) must underly the reduced cardiotoxicity.

Acknowledgement

The authors wish to thank Dr. F. Arcamone from Farmitalia Carlo Erba, Milan, Italy, for his generous gift of the anthracycline analogs which were used in this study.

TABLE I

Oxygen consumption (as a measure for redox cycling), production of thiobarbituric acid-reactive material in liver microsomes, and therapeutic activity and toxicity of the anthracyclines ①, ③, ⑤, ⑥, ⑦ and ⑧

drug		oxygen consumption*	stimulation of production of thiobarbituric acid material**	therapeutic activity and toxicity***
daunorubicin	①	+	+	
doxorubicin	③	+	+	
4-demethoxydaunorubicin	⑤	+	+[a]	reduced cardiotoxic index, higher potency as compared with 1
4'-O-methyldoxorubicin	⑥	+	+[b]	slightly higher potency, reduced cardiotoxicity (mice) as compared with 3
4'-deoxydoxorubicin	⑦	+	+	higher potency, reduced cardiotoxicity and cardiotoxic index as compared with 3
4'-epidoxorubicin	⑧	+	+[b]	equal potency, reduced cardiotoxicity as compared with 3

*this study: + means stimulation.

**this study: + means stimulation. [a]significantly higher with $p < 0.001$ from the parent compound. [b]significantly higher with $p < 0.01$ from the parent compound.

***from ref. 22.

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