Free Rad. Res. Comms., Vol 1, No. 6, pp. 369-378
8755-0199/86/0106-0369 $25-00/0

Printed in Great Britain

NON-ENZYMIC LIPID PEROXIDATION IN MICROSOMES AND MICROSOMAL PHOSPHOLIPIDS INDUCED BY ANTHRACYCLINES

LYDI STERRENBERG, RINETTE H.M. JULICHER, PETER A.L. GOOSSENS, AALT BAST and JAN NOORDHOEK

Dept. of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

(Received August 1, 1985, in final form October 23, 1985)

The stimulation of non-enzymic lipid peroxidation by doxorubicin, daunorubicin and 7 derivatives was investigated in extracted microsomal phospholipids and in intact microsomes.

Evidence was obtained for the necessity of a free amino-sugar moiety for a stimulative effect on lipid peroxidation. Binding of anthracyclines to RNA (which is present in microsomes) was inhibitory towards stimulation.

Drugs that stimulated lipid peroxidation in a non-enzymic system with extracted phospholipids also were stimulative in an enzymic, NADPH-dependent, microsomal system. They were not always effective in intact microsomes without the enzymic system.

The role of the enzymic system in the stimulation of anthracycline induced lipid peroxidation is thought to be the reduction of iron ions rather than the stimulation of oxygen radical production via the anthracyclines.

Key words: anthracyclines, iron ions, lipid peroxidation, microsomes, microsomal phospholipids

INTRODUCTION

Several years ago it was found that quinone compounds like adriamycin could undergo redox-cycling, by enzymic activation to a semiquinone and subsequent reoxidation by oxygen.[1,2,3] This redox-cycling process leads to the formation of superoxide anion radicals. Other forms of activated oxygen species that initiate lipid peroxidation were thought to be formed by dismutation and via iron-dependent reactions. The formation of these reactive oxygen radicals and the attack on unsaturated membrane lipids, have been mentioned as a factor in the development of the cardiomyopathy which can occur on adriamycin-treatment.[4,5]

We previously found in a study with several anthracycline analogs that all these compounds stimulated oxygen consumption in microsomes.[6] Under the conditions used (when oxygen uptake via lipid peroxidation is blocked by EDTA) the oxygen consumption represented enzymic activation and consequent formation of oxygen radicals. Yet, of the compounds tested, two did not stimulate microsomal NADPH-

dependent lipid peroxidation (aglycones of doxorubicin and daunorubicin). From these experiments it was concluded that enzymic activation of the anthracyclines does not necessarily lead to lipid peroxidation.

Recently growing evidence has been collected on a non-enzymic stimulation of lipid peroxidation.[7,8,9] This stimulation has been suggested to be caused by an intermolecular electron transfer mechanism within an adriamycin-iron co-ordination complex.[7,8,910]

In this paper we investigate this non-enzymic stimulation of lipid peroxidation with the anthracyclines mentioned before. In addition AD-32, an adriamycin-derivative which has been shown to be less cardiotoxic, has been included in this study.

The stimulative effect was tested both with extracted microsomal phospholipids and with intact microsomes.

MATERIALS AND METHODS

Materials

$NADP^+$, RNA (type IV, calf liver), DNA (calf thymus, type I) and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, USA.

Fe^{2+} (as $FeSO_4.7H_2O$) was from Merck, Darmstadt, FRG and Fe^{3+} (as $Fe(NO_3)_3.9H_2O$) from Baker Chemicals BV, Deventer, The Netherlands. Water with a low defined concentration of iron-ions (Analar®) was obtained from BDH-Chemicals Ltd., Poole, England.

Daunorubicin.HCl (*1*), daunorubicinone (*2*), doxorubicin.HCl (*3*), doxorubicinone (*4*), 4-demethoxydaunorubicin.HCl (*5*), 4′-O-methyldoxorubicin.HCl (*6*), 4′-deoxydoxorubicin.HCl (*7*), 4′-epidoxorubicin.HCl (*8*) and N-trifluoroacetyldoxorubicin-14-valerate (AD-32, *9*) were all gifts from dr. F.F. Arcamone, Farmitalia Carlo-Erba, Milan, Italy. The structures of compounds *1–8* were depicted in a previous paper[6]; figure 1 shows the structure of compound *9*.

All other reagents used were of analytical grade purity.

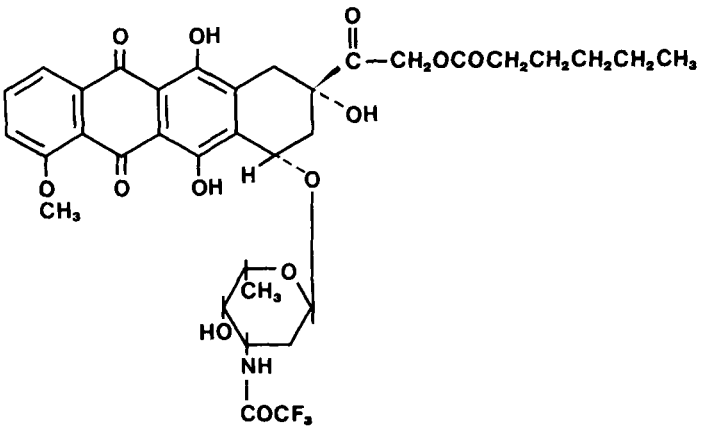

FIGURE 1 Structure of AD-32 (compound *9*).

Methods

Preparation of microsomes. Microsomes were prepared from 3–5 livers from male Wistar rats (200–250 g, TNO, Zeist, The Netherlands). Therefore, the rats were decapitated and livers were removed. The livers were homogenized (w/v: 1/2) in cold 50 mM phosphate buffer pH 7.4 containing 0.1 mM EDTA. Microsomes were prepared by centrifugation as described before.[11] The microsomal pellet was suspended in phosphate buffer and stored at −80°C. Before use, the microsomes were diluted with 50 mM Tris, 150 mM KCl, pH 7.4 in Analar water, washed twice by repeated centrifugation, and resuspended in the Tris-buffer.

Extraction of microsomal lipids. Microsomal lipids were extracted as described by Folch *et al.*[12] Briefly, lipids from ± 30 mg microsomal protein in 1 ml were extracted with 5 ml methanol/chloroform (1:2 v/v). After centrifugation the chloroform layer was taken and the chloroform was evaporated by heating the lipid-extract at 50°C under nitrogen. Extracted lipids were sonified with 5 ml Tris-HCl buffer for ± 10 min, giving a milky-white suspension. Before use the lipid-in-Tris-suspension was centrifuged at room temperature for 5 min at 1500 g. This suspension was finally used twice diluted.

Peroxidation conditions. Non-enzymic peroxidation of extracted microsomal lipids or intact microsomes was induced as follows. Incubates of 3 ml volume contained either 1 mg/ml protein or the above mentioned microsomal lipid extract, and 50 μM anthracyclines, solubilized in DMSO (final DMSO concentration 1% v/v). Control incubates contained microsomal phospholipids plus DMSO. When present, the RNA concentration was 1 mg/ml. The peroxidation process was started with the addition of a concentrated iron solution. Fe^{2+} and Fe^{3+} solutions were freshly prepared in 10^{-4} M HCl. The incubation was at 37°C in a shaking water bath. Air was freely admitted.

NADPH-dependent lipid peroxidation was induced in microsomes as described[11], using the NADPH-regenerating system of Mimnaugh *et al.*[5] The concentration glucose-6-phosphate dehydrogenase was 0.85 U/ml. The total volume was 3 ml.

Assessment of lipid peroxidation. For the assessment of lipid peroxidation at different times samples of 0.5 ml were taken. Lipid peroxidation was measured as thiobarbituric acid reactive material essentially as described by Folch *et al.*[13] For experiments with microsomes the lipid peroxidation is expressed as nmol malondialdehyde (MDA)/mg protein, using a molar extinction coefficient for MDA of 1.56×10^5 M^{-1} cm^{-1}.

Extraction of coloured material in butanol was applied with extracted phospholipids since these were not precipitable with trichloroacetic acid. Lipid peroxidation in extracted lipids was then expressed as "% of the maximal MDA formation". The maximal production of thiobarbituric acid was assessed by incubation of the extracted phospholipids (at the same concentration as used in the other peroxidation experiments) with 5 μM Fe^{2+} and 0.1 mM ascorbic acid for 1 hours at 37°C.

Other assays. Protein was measured with the Folin phenol reagent.[14] RNA in microsomes was measured according to Karsten.[15] The microsomal preparations were found to contain ± 6 mg RNA/100 mg protein.

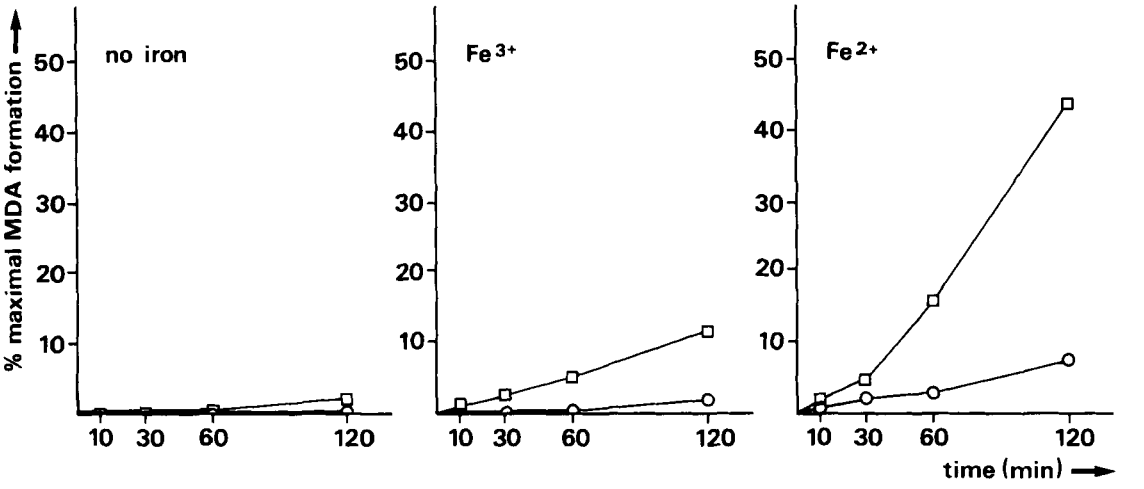

FIGURE 2 Stimulation of lipid peroxidation by doxorubicin (*3*) in extracted microsomal phospholipids in the absence of an enzymic system. No iron, 13 μM Fe^{3+} or 13 μM Fe^{2+} was added.
□—□ lipid peroxidation in the presence of 50 μM doxorubicin
○—○ lipid peroxidation in the absence of anthracycline
Maximal lipid peroxidation corresponds with a ΔA_{532}/ml of 8,1.

RESULTS

Figures 2 and 3 summarize the results of experiments using extracted phospholipids. Figure 2 shows that doxorubicin slightly enhanced MDA formation in the absence of iron ions. Addition of ferric and ferrous ions (5 μM) further enhanced MDA formation, the latter being most effective. For further experiments a combined addition of ferrous ions (5 μM) and the anthracyclines was used.

Figure 3 shows that under these conditions compounds *1*, *3*, *5*, *6*, *7* and *8* significantly enhanced peroxidation of the phospholipids, whereas compounds *2*, *4* (aglycones) and *9* (blocked amino-group in the sugar moiety) were ineffective at the chosen concentration (50 μM).

The same figure also shows the effect of RNA (which is present in microsomal preparations) on the anthracycline-Fe^{2+} induced lipid peroxidation. Enhanced thiobarbituric acid (TBA) reactivity was found due to addition of RNA in the control; a similar increase in TBA-reactivity due to addition of RNA was found for compounds *2*, *4* and *9*. These drugs again did not stimulate peroxidation as compared with the RNA containing control. RNA addition decreased the stimulatory effect of all other compounds slightly or pronounced and led to a loss in significance of stimulation for drugs *1* and *8*.

Further studies were performed using hepatic microsomes instead of extracted phospholipids. In figure 4 the effects of compounds *1–9* on non-enzymic lipid peroxidation is presented. Only compound *9* stimulated the process whereas the aglycone *2* inhibited peroxidation.

Finally, in microsomes a comparison in more detail was made for doxorubicin (*3*) between the enzymic, NADPH-dependent and the non-enzymic system. The com-

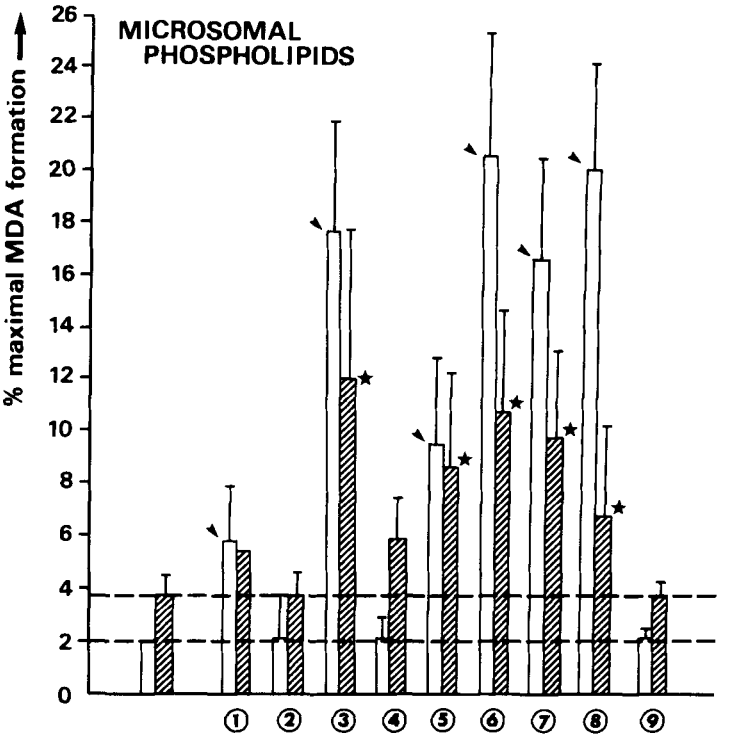

FIGURE 3 Non-enzymic lipid peroxidation in extracted microsomal phospholipids. Control: no anthracyclines added. Numbers refer to the drug added (50 μM). The concentration of added Fe^{2+} was 5 μM. Lipid peroxidation was allowed to proceed for 2 hours. For other details see Materials and Methods. N = 4.
□ lipid peroxidation in the absence of RNA
▨ effect in the presence of 1 mg/ml RNA
Significancies were calculated using the Student's t-test (t-dependent). Significancies are indicated for the effect of the drugs compared with the appropriate control.
▶ Significant with $p < 0.05$, system without RNA.
* Significant with $p < 0.05$, system with 1 mg RNA/ml.
Maximal lipid peroxidation corresponds with a ΔA_{532}/ml of 8,1.

parison was made in buffer without added iron ions and in buffer to which Fe^{2+}-ions were added to a concentration of 2 μM. From figure 5A it can be seen that non-enzymic lipid peroxidation in microsomes was inhibited by doxorubicin. When $NADP^+$ (1.9 mM) was added (figure 5B), which is a constituent of the NADPH-regenerating system, and the small amount of Fe^{2+}-ions, doxorubicin slightly stimulated TBA-reactivity. The NADPH-regenerating system augmented the TBA-reactivity (figure 5C). Only in the case that no extra Fe^{2+}-ions were added, the enzymic system potentiated the stimulative effect of adriamycin found in the non-enzymic system.

DISCUSSION

The objective of this study was to investigate whether activation of anthracyclines and

the consequent production of oxygen radicals are necessary for, or add to, the peroxidative effect of the anthracyclines in microsomal membranes. We also wanted to know whether the aglycones of doxorubicin and daunorubicin were able to stimulate non-enzymically lipid peroxidation. These aglycones are not stimulative in enzymic, NADPH-dependent lipid peroxidation in microsomes[6], but have the structural features for complex-forming with iron ions.[16,17] This means that these drugs would have the potential of intermolecular electron transfer within the iron-adriamycin complex and should stimulate non-enzymic lipid peroxidation.

The experiments were performed with Fe^{2+}-ions since they were found to be a better stimulator of lipid peroxidation then Fe^{3+}-ions (figure 2). Also in NADPH-dependent microsomal lipid peroxidation continuous enzymic reduction of Fe^{3+} to Fe^{2+} will occur. This favours the NADPH-dependent lipid peroxidation in microsomes compared to the non-enzymic system, since the Fe^{2+}-concentration limits the stimulative effect.

With extracted phospholipids a picture of stimulating capacity for compounds *1–8* emerged that qualitatively corresponded to that obtained in microsomes with a NADPH-regenerating system.[6] That is only compounds *1*, *3*, *5*, *7* and *8* significantly stimulated the MDA production. Thus, non-enzymic lipid peroxidation in extracted phospholipids seems to accurately predict the stimulative capacity of anthracyclines in enzymic microsomal lipid peroxidation. The relatively low stimulating effect obtained for daunorubicin *1* and the daunorubicin derivative *5* was due to a lag time before the stimulating effect appeared (not shown). Such a lag time was not found for the doxorubicin derivatives. From the results with AD-32 and with the aglycones it may be inferred that the presence of the aglycone part of the molecule per se is not sufficient for stimulation of lipid peroxidation.

We previously found spectral evidence for a binding of anthracyclines to microsomal ribosomal RNA.[6] When RNA was added to the phospholipids, enhanced MDA production was found with the aglycones (*2* and *4*) and with AD-32 (*9*). Since a similar enhancement of MDA production by RNA was found in the control, it can be inferred that RNA addition did not affect the aglycone- or AD-32-stimulated lipid peroxidation. All three drugs also have an impaired nucleic acid binding, due to the lack of a sugar-moiety (the aglycones) or due to a sugar-moiety with a blocked amino-group (AD-32).[16] With other anthracyclines, addition of RNA inhibited the stimulating effect of the drugs. These anthracyclines all possess a sugar-moiety with a free amino-group. Via this group they should be able to bind to RNA. Thus, binding to RNA apparently reduced the stimulating effect of drugs on lipid peroxidation. Binding to nucleic acid has been reported to block enzymic activation as well[18,19]. Our data also suggest that in addition to the aglycone part of the molecule a free amino-sugar group is needed for stimulation of MDA production: only those compounds that possess such a group enhanced non-enzymic lipid peroxidation.

The results with extracted lipids (figure 4) were no model for non-enzymically induced lipid peroxidation in intact microsomal membranes. Most drugs lost their lipid peroxidation-stimulatory capacity in microsomal membranes. The non-enzymic stimulation of lipid peroxidation in microsomes also differed from the stimulation in microsomes in the presence of an enzyme-system. E.g. an inhibition (though not significant) was found for doxorubicin (*3*) in the non-enzymic system in microsomes whereas this drug stimulated lipid peroxidation in microsomes in the presence of an NADPH-dependent enzymic system and in extracted phospholipids. Using Fe^{3+}-ions instead of Fe^{2+}-ions did not change this (not shown).

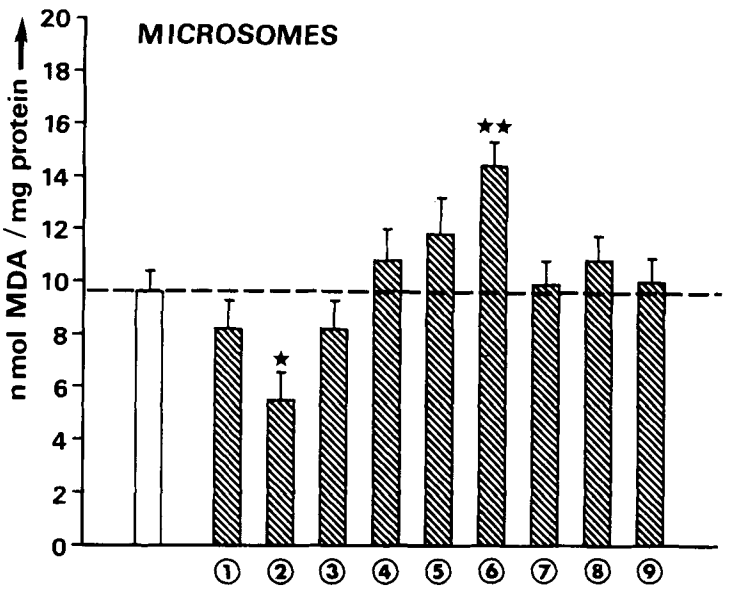

FIGURE 4 Non-enzymic lipid peroxidation in intact microsomal membranes. Microsomes (1 mg protein/ml) were incubated with 5 μM Fe^{2+} and 0 (□) or 50 μM anthracycline (▨) for 2 hours. The numbers refer to the drug used. Significancies were computed with Student's t-test (t-dependent). Significancies are indicated for the effect of the drug compared to the control.
* $p < 0.05$; ** $p < 0.001$; $N = 5$.
1 nmol MDA/mg protein corresponds with a ΔA_{532}/ml of 0.053.

A slightly stimulative effect of doxorubicin in non-enzymic lipid perodixation in microsomes was however observed when $NADP^+$ was added to the microsomes (figure 5B). $NADP^+$ is a constituent of the regenerating system used in NADPH-dependent microsomal lipid peroxidation. Though also the control was stimulated by the addition of $NADP^+$, the effect on doxorubic induced non-enzymic lipid peroxidation was greater (at 120 min the stimulation for the control was 3x, whereas the stimulation for adriamycin was 10x). How $NADP^+$ affects the stimulative activity of adriamycin is not known. $MgCl_2$, present in the regenerating system in a concentration of 9 mM, did not affect the stimulation of lipid peroxidation by adriamycin. Also glucose-6-phosphate dehydrogenase, when denatured, had not effect (not shown).

The enzymic system (figure 5C) enhanced MDA production compared to the non-enzymic system in the presence of $NADP^+$ (figure 5B). However it potentiated the stimulatory effect of doxorubicin only when no extra iron was added (thus at an iron concentration, contaminating the buffer). As we also reported before[6] at higher iron concentrations the stimulative effect of adriamycin diminishes.

On the basis of previous work[6,11] and of this study we think that the contribution of the NADPH-regenerating system in the induction of lipid peroxidation is due to the generation of limited amounts of Fe^{2+}-ions. The reduction of Fe^{3+}-ions to Fe^{2+}-ions is direct i.e. by the enzyme, or could proceed indirectly via the aglycone part of the anthracyclines (figure 6). The latter reaction is likely to be favoured under hypoxic conditions.[20,21] It is only when the enzymic reduction of Fe^{3+}-ions is promoted by the indirect way and when a free amino-group in the anthracycline sugar moiety is present

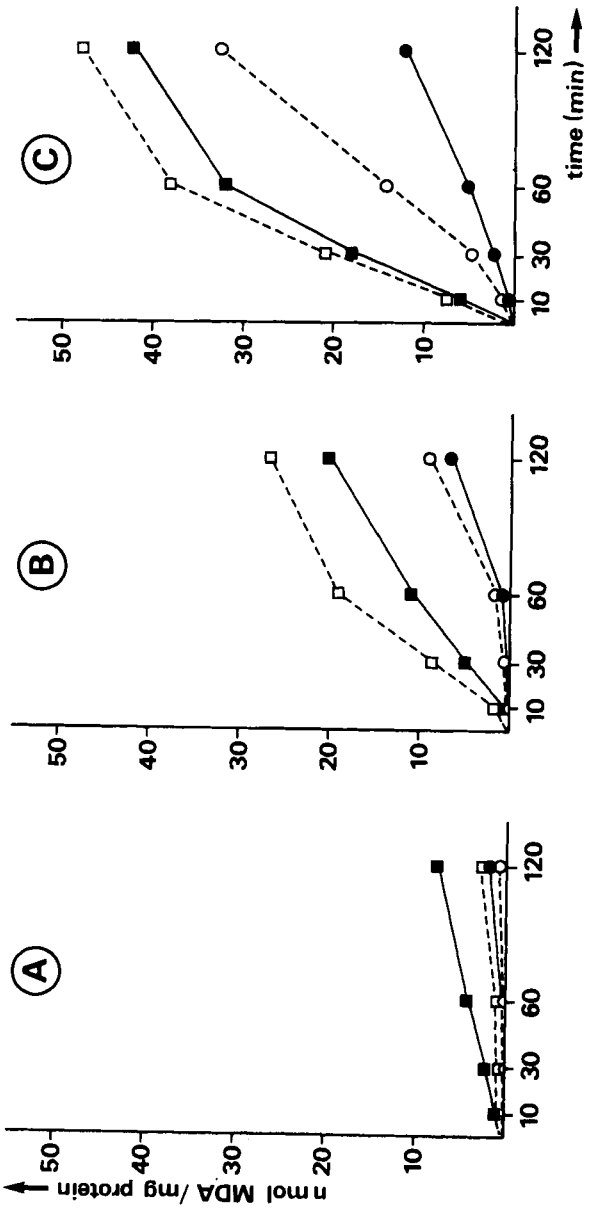

FIGURE 5 Lipid peroxidation in microsomes in the absence (A, B) or presence (C) of a NADPH-regenerating system. In B $NADP^+$ (1.9 mM) is extra present as compared to A. The microsomal concentration was 1 mg protein/ml. N ≈ 2.
●—— no iron ions added, no doxorubicin
○----- no iron ions added, 50 μM doxorubicin
■—— Fe^{2+} ions added to a concentration 2 μM, no doxorubicin
□----- Fe^{2+} ions added to a concentration of 2 μM, 50 μM doxorubicin
1 nmol MDA/mg protein corresponds with a ΔA_{532}/ml of 0.053.

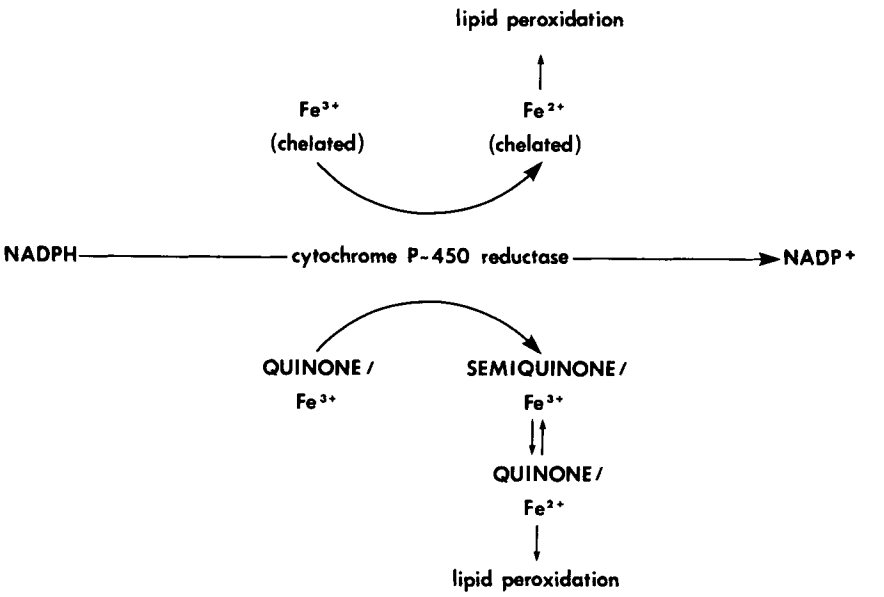

FIGURE 6 Proposed contribution of microsomal NADPH-dependent enzymic activity (cytochrome P450-reductase) to microsomal lipid peroxidation. Enzymic activity results in the reduction of Fe^{3+} to Fe^{2+} either directly, or indirectly via the aglycone part of the anthracycline. In the latter situation an adriamycin-iron complex might be involved, with intramolecular electron transfer. Alternatively, the semiquinone form of the aglycone-part might reduce free iron.

that lipid peroxidation is stimulated by the anthracyclines. The aglycones, which we found to be reducable[6], and AD-32 lack this latter required structural feature.

Acknowledgements

The authors wish to thank Dr. F.F. Arcamone for his generous gift of the anthracycline-derivatives used in this study, Mr. P. van Dorp van Vliet and P. Chan for making the drawings and Ms. A. van Putten for typing the manuscript.

References

1. K. Handa and S. Sato, *Gann*, **67**, 523, (1976).
2. J. Goodstein and P. Hochstein, *Biochem. Biophys. Res. Comm.*, **77**, 797, (1977).
3. N.R. Bachur, S.L. Gordon, M.V. Gee and H. Kon, *Proc. Natl. Acad. Sci. USA*, **76**, 954, (1979).
4. C. Myers, W.P. McGuire, R.H. Liss, I. Ifrim, K. Grotzinger and R.C. Young, *Science*, **19**, 165, (1977).
5. E.G. Mimnaugh, M.A. Trush and T.E. Gram, *Biochem. Pharmacol.*, **30**, 2797, (1981).
6. L. Sterrenberg, R.H.M. Julicher, P.A.L. Goossens, A. Bast and J. Noordhoek, *Free Radical Res. Comm.* (in press).
7. K. Sugioka and M. Nakano, *Biochim. Biophys. Acta*, **713**, 333, (1982).
8. H. Nakano, K. Ogita, J.M.C. Gutteridge and M. Nakano, *FEBS Lett.*, **166**, 232, (1984).

9. J.M.C. Gutteridge, *Biochem. Pharmacol.*, **33**, 1725, (1984).
10. J.L. Zweier, *J. Biol. Chem.*, **259**, 6056, (1984).
11. L. Sterrenberg, R.H.M. Julicher, A. Bast and J. Noordhoek, *Toxicol. Lett.*, **22**, 153, (1984).
12. J. Folch, M.M. Lees and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497, (1957).
13. J.A. Buege and S.D. Aust, in Methods in Enzymology (Acad. Press: New York) vol. 3, p. 302, (1978).
14. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265, (1951).
15. U. Karsten and A. Wollenberger, *Anal. Biochem.*, **77**, 464, (1977).
16. E. Calendi, A. Dimarco, M. Reggiani, B. Scarpinato and L. Valentini, *Biochim. Biophys. Acta*, **103**, 25, (1965).
17. H. Eliot, L. Gianni and C. Myers, *Biochemistry*, **23**, 928, (1984).
18. R.J. Youngman and E.F. Elstner, *Arch. Biochem. Biophys.*, **231**, 424, (1984).
19. D.A. Rowley and B. Halliwell, *Biochim. Biophys. Acta*, **761**, 86, (1983).
20. J.M.C. Gutteridge, G.J. Quinlan and S. Wilkins, *FEBS Letters*, **167**, 37, (1984).
21. J. Butler, B.M. Hoey and A.J. Swallow, *FEBS Letters*, **182**, 95, (1985).