Biochemical Pharmacology, Vol. 35, No. 23, pp. 4327–4335, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

A POSSIBLE ROLE FOR MEMBRANE LIPID PEROXIDATION IN ANTHRACYCLINE NEPHROTOXICITY*

EDWARD G. MIMNAUGH,† MICHAEL A. TRUSH‡ and THEODORE E. GRAM

Pharmacology and Toxicology Section, Laboratory of Experimental Therapeutics and Metabolism, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

(*Received* 21 *October* 1985; *accepted* 7 *May* 1986)

Abstract—Adriamycin causes both glomerular and tubular lesions in kidney, which can be severe enough to progress to irreversible renal failure. This drug-caused nephrotoxicity may result from the metabolic reductive activation of Adriamycin to a semiquinone free radical intermediate by oxidoreductive enzymes such as NADPH–cytochrome P-450 reductase and NADH-dehydrogenase. The drug semiquinone, in turn, autoxidizes and efficiently generates highly reactive and toxic oxyradicals. We report here that the reductive activation of Adriamycin markedly enhanced both NADPH- and NADH-dependent kidney microsomal membrane lipid peroxidation, measured as malonaldehyde by the thiobarbituric acid method. Adriamycin-enhanced kidney microsomal lipid peroxidation was diminished by the inclusion of the oxyradical scavengers, superoxide dismutase and 1,3-dimethylurea, and by the chelating agents, EDTA and diethylenetriamine-pentaacetic acid (DETPAC), implicating an obligatory role for reactive oxygen species and metal ions in the peroxidation mechanism. Furthermore, the inclusion of exogenous ferric and ferrous iron salts more than doubled Adriamycin-stimulated peroxidation. Lipid peroxidation was prevented by the sulfhydryl-reacting agent, *p*-chloromercuribenzenesulfonic acid, by omitting NAD(P)H, or by heat-inactivating the kidney microsomes, indicating the requirement for active pyridine-nucleotide linked enzymes. Several analogs of Adriamycin as well as mitomycin C, drugs which are capable of oxidation–reduction cycling, greatly increased NADPH-dependent kidney microsomal peroxidation. Carminomycin and 4-demethoxydaunorubicin were noteworthy in this respect because they were three to four times as potent as Adriamycin. In isolated kidney mitochondria, Adriamycin promoted a 12-fold increase in NADH-supported (NADH-dehydrogenase-dependent) peroxidation. These observations clearly indicate that anthracyclines enhance oxyradical-mediated membrane lipid peroxidation *in vitro*, and suggest that peroxidation-caused damage to kidney endoplasmic reticulum and mitochondrial membranes *in vivo* could contribute to the development of anthracycline-caused nephrotoxicity.

The anthracycline antitumor drug Adriamycin causes severe nephrotoxicity in a variety of experimental animals [1–5], and under certain conditions, Adriamycin may be nephrotoxic to humans [6]. Adriamycin-associated nephrotoxicity includes both glomerular and tubular lesions [7], and the resulting drug-induced chronic glomerulonephritis may progress to irreversible kidney failure [8]. Although renal toxicity in humans is not usually considered to be dose-limiting to chemotherapy with Adriamycin, life-threatening nephrotoxicity could result from aggressive Adriamycin treatment in patients with underlying kidney disease and compromised renal function. Alternatively, it could become manifested by extending Adriamycin therapy beyond the cumulative cardiotoxic dose threshold of 500–600 mg/m^2 [9] through the use of cardioprotective agents such as *N*-acetylcysteine [10] or α-tocopherol [11]. Furthermore, other anthracyline antitumor drugs in addition to Adriamycin appear to cause severe renal toxicity. For example, carminomycin is a potent nephrotoxin, causing biochemical and histological evidence of nephrotoxicity in animals at comparatively low doses [12], and mitomycin C, another quinone-containing anticancer drug, has been reported to be nephrotoxic to both animals [13, 14] and humans [15].

The molecular mechanisms by which anthracyclines cause renal damage are unknown; however, numerous studies support the suggestion that the host toxicities of anthracyclines may be the consequence of oxidative stress, that is, oxidation and cross-linking of cellular thiols [16] and membrane lipid peroxidation [17, 18]. We have reported previously that Adriamycin enhances the peroxidation of unsaturated membrane phospholipids in hepatic microsomal [19], mitochondrial [20] and nuclear subcellular fractions [21], as well as in microsomes from hearts [22]. Considerable evidence suggests that this process results from the enzymatic half-reduction of the Adriamycin quinone to a reactive semiquinone free radical intermediate by membrane-bound NADPH–cytochrome P-450 reductase (EC 1.6.2.4)

* These results were presented in part at the Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, August 7–11, 1983, in Philadelphia, PA.

† Author to whom all correspondence should be addressed at the National Cancer Institute, NIH, Bldg. 10, Rm. 6N119, Bethesda, MD 20892.

‡ Present address: Department of Environmental Health Sciences, Division of Experimental Pathology and Toxicology, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205.

[23–25] and by mitochondrial NADH-dehydrogenase (EC 1.6.99.3) [26]. In the presence of oxygen, the Adriamycin semiquinone radical spontaneously autoxidizes and participates in an oxidation–reduction cycle which generates superoxide free radical [27, 28] and secondary reactive oxygen species derived from superoxide including perhydroxyl radical [29], hydrogen peroxide [30] and hydroxyl radical [31]. This oxyradical cascade then accelerates the peroxidation of polyunsaturated lipids [19, 32].

Because the peroxidation of membrane lipids causes deleterious alterations in cellular membrane structure and function that can ultimately lead to cytotoxicity [33, 34], we hypothesized that Adriamycin free radical-initiated, oxyradical-mediated membrane lipid peroxidation could be a possible biochemical mechanism of Adriamycin-caused nephrotoxicity. A recent report provided evidence that the reperfusion of ischemic kidney resulted in the production of toxic oxyradicals through the action of xanthine oxidase on xanthine, and the resulting renal injury was considered to be a consequence of membrane lipid peroxidation [35].

To test the feasibility of the lipid peroxidation mechanism of Adriamycin nephrotoxicity, we have investigated the effects of Adriamycin *in vitro* on enzyme-mediated peroxidation of unsaturated membrane phospholipids in microsomes and mitochondria isolated from kidney. Through the use of specific chemical and enzymatic oxyradical scavengers, we have implicated the obligatory participation of both superoxide and hydroxyl free radicals in the drug-enhanced peroxidation process.

METHODS

Animals and drugs. Adult male Sprague–Dawley rats, 200–300 g (Taconic Farms, Germantown, NY), and male CDF_1 mice, 20–25 g (Flow Laboratories, Dublin, VA), were fed Purina laboratory chow and water *ad lib.* and were not used for at least 2 weeks after delivery to the laboratory. All drugs used in this study were obtained from the Division of Cancer Treatment, NCI, NIH (Bethesda, MD). Just prior to use, they were dissolved in oxygenated (bubbled with 100% oxygen for at least 10 min) 150 mM KCl–50 mM Tris–HCl buffer, pH 7.4 (KCl–Tris), and the drug solutions were protected against light exposure with aluminum foil.

Isolation of microsomes and mitochondria. Kidneys from three to four rats or ten to twelve mice were removed from animals killed by cervical fracture, rinsed in KCl–Tris buffer, decapsulated, and combined to make one sample. In this study no attempt was made to separate the renal cortex from the medulla. For microsomal fractions, the kidneys were homogenized in KCl–Tris buffer and isolated by differential centrifugation as described by Gram *et al.* [36]. Mitochondria were isolated by centrifugation following homogenization in 0.25 M sucrose: 25 mM Tris : 1 mM EDTA : 1% (w/v) BSA* isolation solution as previously described [20, 37]. Following the final pelleting of the kidney mitochondria in the isolation solution, they were resuspended and repelleted by centrifugation (11,500 $g \times 6$ min) in KCl–Tris buffer three additional times to remove the sucrose, EDTA and bovine serum albumin.

Assay for lipid peroxidation. Kidney microsomes or mitochondria (1 mg protein/ml) were incubated in oxygenated KCl–Tris buffer with Adriamycin (100 μM) or other drugs and either ascorbic acid (0.5 mM), NADH (2.5 mM), NADPH (2.5 mM) or an NADPH-generating system consisting of NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 units/ml) and magnesium chloride (4.3 mM), unless otherwise noted. In several experiments, exogenous iron salts (ferric chloride and ferrous sulfate) were added to the reaction mixtures subsequent to adding Adriamycin. The iron salts were dissolved just before use in nitrogen-bubbled glass distilled water. Incubations were conducted under a 100% oxygen atmosphere in the dark at 37°, usually for 60 min. This reaction mixture has been described in detail previously for both Adriamycin-stimulated microsomal [22] and mitochondrial lipid peroxidation [20]. The reactions were terminated by adding cold trichloroacetic acid, and lipid peroxidation was quantitated spectrophotometrically as malonaldehyde equivalents following the addition of 2-thiobarbituric acid, as described initially by Bernheim *et al.* [38] and subsequently modified for use in our laboratory [19, 22].

Other assays. Protein was measured by the method of Lowry *et al.* [39], with bovine serum albumin as the standard. The rates of NADPH and NADH oxidation were followed spectrophotometrically by monitoring their absorption at 340 nm. NADPH–cytochrome P-450 reductase activity was determined by the method of Williams and Kamin [40] with cytochrome *c* as the acceptor substrate, NADH–cytochrome b_5 reductase activity was estimated by measuring the reduction of ferricyanide [41], and membrane α-tocopherol was determined fluorometrically as described by Taylor *et al.* [42]. All assays were performed on at least three samples of kidney microsomes or mitochondria.

Data were analyzed statistically by Student's *t*-test [43], and differences between mean values at $P < 0.05$ were considered to be significant.

RESULTS

Kidney microsomal oxidoreductase activities. Enzyme-catalyzed NAD(P)H-dependent lipid peroxidation in kidney subcellular fractions has not been studied extensively, but several reports have indicated NADPH-induced lipid peroxidation in renal microsomes to be qualitatively similar to, although quantitatively less than, peroxidation in hepatic microsomes [44, 45]. It is also known that NADPH–cytochrome P-450 reductase activity in kidney microsomes from common laboratory species varies from 30 to 70% of the corresponding activity found in liver microsomes [46]. Therefore, at the outset, we measured the activities of several kidney microsomal oxidoreductase enzymes which could potentially acti-

* Abbreviations: BSA, bovine serum albumin; DETPAC, diethylenetriamine-pentaacetic acid; MDA, malonaldehyde; and *p*-CMB, *p*-chloromercuribenzene sulfonic acid.

Table 1. Comparison of oxidoreductase enzyme activities in kidney microsomes from mice and rats*

	NADPH–Cytochrome P-450 reductase†	NADH–Cytochrome b_5 reductase‡	NADH–Cytochrome *c* reductase†
Mouse	172 ± 11§	3920 ± 190	1070 ± 82
Rat	75 ± 8	2170 ± 260	206 ± 3

‡ Microsomes were incubated with the appropriate cofactors and substrates, and the enzyme activities were measured spectrophotometrically using the initial reaction rates. Adriamycin at 100 μM did not alter significantly the enzyme activities.

† Expressed as nmoles of cytochrome *c* reduced/mg protein/min at 37°.

‡ Expressed as nmoles of ferricyanide reduced/mg protein/min at 37°.

§ Data are expressed as the mean ± SD for N = 3–6.

vate Adriamycin. A comparison of the activities of several oxidoreductases in mouse and rat kidney microsomes is shown in Table 1. NADPH–cytochrome P-450 reductase, NADH–cytochrome b_5 reductase and NADH–cytochrome *c* reductase activities were found to be from 2- to 5-fold higher in mouse kidney microsomes than in microsomes from rats. Uniformly, the measurements of the reductase enzyme activities were not altered significantly by the presence of Adriamycin at 100 μM (data not presented).

NAD(P)H oxidation. The effects of Adriamycin on the abilities of rat and mouse kidney microsomes to catalyze NADPH and NADH oxidation are shown in Table 2. The endogenous rate of NADPH oxidation in the presence of kidney microsomes from either species was increased 6-fold by Adriamycin (100 μM). In contrast, in the presence of Adriamycin, there was an apparent trend in the rate of NADH oxidation toward lower values, but the difference was not significant. Adriamycin did not oxidize either NADPH or NADH in the absence of kidney microsomes or in the presence of heat-denatured microsomes (data not shown).

Source of reducing equivalents for lipid peroxidation. The data in Table 3 show that the preferred biological reducing agent for enzyme-catalyzed lipid peroxidation in kidney microsomes from either rats or mice was NADPH. (In this experiment concentration of the cofactors was arbitrarily set at 2.5 mM.) In incubations conducted in the absence of NAD(P)H or in reaction mixtures that included NAD(P)H but were stopped at zero time, there was negligible malonaldehyde measured with either rat or mouse kidney microsomes (0.5 to 2 nmoles MDA/mg protein). All lipid peroxidation data have been corrected for zero-time values under the various experimental conditions. The inclusion of Adriamycin (100 μM) resulted in a 5- to 7-fold enhancement of NADPH-dependent lipid peroxidation in kidney microsomes, which correlated well with the nearly proportional increase which was measured in the rate of NADPH oxidation caused by Adriamycin. The effects of NADPH and NADH in combination (both pyridine nucleotides at 2 mM) had additive rather than synergistic effects on peroxidation, in either the absence or presence of Adriamycin (data not presented). Although NADH supported lipid peroxidation in mouse kidney microsomes reasonably well, in contrast, it was ineffective as a source of reducing equivalents with rat kidney microsomes (Table 3). The NADPH-generating system, which maintained the concentration of NADPH throughout the incubation period, was superior to NADPH alone.

Ascorbic acid-promoted microsomal lipid peroxidation, a nonenzymatic chemical process which is believed to depend upon the reduction of trace amounts of ferric iron [47, 48] to generate superoxide anion radical [49], was inhibited by Adriamycin (Table 3). Ascorbate-dependent peroxidation of kidney microsomes was nearly abolished by Adriamycin at 200 μM.

When rat and mouse kidney lipid peroxidation

Table 2. Effects of Adriamycin on the rates of NADPH and NADH oxidation catalyzed by kidney microsomes*

	(nmoles NADPH oxidized/mg protein/min)		(nmoles NADH oxidized/mg protein/min)	
Source of microsomes	Endogenous	Adriamycin (100 μM)	Endogenous	Adriamycin (100 μM)
Mouse	6.3 ± 0.4	39 ± 1†	159 ± 37	107 ± 8
Rat	3.9 ± 0.5	26 ± 1†	193 ± 41	145 ± 33

* Microsomes and either NADPH or NADH were incubated at 37° in KCl–Tris buffer, pH 7.4, and the oxidation of the pyridine nucleotides was monitored spectrophotometrically at 340 nm.

† Significantly different from values (mean ± SD) measured in the absence of Adriamycin at $P < 0.05$, N = 3–6.

Table 3. Comparison of the source of reducing equivalents for Adriamycin-stimulated kidney microsomal lipid peroxidation*

Species	Reducing agent	Microsomal lipid peroxidation (nmoles MDA equivalents/mg protein/60 min) Endogenous	Adriamycin (100 μM)
Mouse	NADPH (2.5 mM)	4.7 ± 0.5†	43.2 ± 2.1‡
	NADPH-generating system§	11.4 ± 0.7	74.6 ± 1.9‡
	NADH (2.5 mM)	2.9 ± 0.3	12.9 ± 0.6‡
	Ascorbic acid (0.5 mM)‖	34.5 ± 1.5	28.0 ± 2.2
Rat	NADPH (2.5 mM)	3.3 ± 0.3	11.1 ± 1.1‡
	NADPH-generating system	5.1 ± 0.4	20.4 ± 1.1‡
	NADH (2.5 mM)	0.2 ± 0.3	2.0 ± 0.1‡

* Mouse or rat kidney microsomes were incubated at 37° in KCl–Tris buffer, pH 7.4, in the absence or presence of Adriamycin at 100 μM. Reactions were started by adding the various reducing agents, and lipid peroxidation was measured by the 2-thiobarbituric acid method.

† Values are expressed as the mean ± SD, N = 3–4.

‡ Significantly greater than values without Adriamycin, $P < 0.001$.

§ The NADPH-generating system consisted of NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 units/ml) and magnesium chloride (4.3 mM) in 0.3 M Tris–HCl buffer, pH 7.4. A 250-μl aliquot of the generating system was added to the incubation mixtures which had a final volume of 1.0 ml.

‖ Neither NADPH nor NADH was included in the reaction mixtures with ascorbic acid. Adriamycin at 200 μM nearly abolished ascorbate-promoted lipid peroxidation (4.0 ± 1.0 nmoles MDA/mg protein/60 min).

activities were normalized to either the microsomal NAD(P)H-dependent oxidoreductase activities (Table 1) or to the rates of NADPH and NADH oxidation (Table 2), it was found that microsomal lipid peroxidation, in general, correlated best with NADPH–cytochrome P-450 reductase activity. In the presence of Adriamycin, lipid peroxidation values for rat kidney ranged from 30 to 80% of the corresponding values for mouse kidney microsomes. This suggests that the magnitude of Adriamycin-enhanced peroxidation in this model system may predominantly depend upon the activities of the enzymes which are capable of reductively activating the drug.

Characterization of lipid peroxidation incubation conditions. To further study the enhancement of kidney microsomal lipid peroxidation by Adriamycin, the incubation conditions were varied with respect to time, the protein and cofactor concentrations, and the concentration of Adriamycin. In these optimization experiments, mouse kidney microsomes were used because they were more susceptible to lipid peroxidation than kidney microsomes from rats (Table 3).

The concentration-dependent effects of Adriamycin and the extent of lipid peroxidation are shown in Fig. 1. In this experiment, the NADPH-generating system was used to ensure that the microsomal flavoprotein, NADPH–cytochrome P-450 reductase, was saturated with respect to NADPH. Although NADPH-dependent kidney microsomal peroxidation was maximally stimulated by Adriamycin at 100 μM, it should be noted that a concentration of the drug as low as 25 μM increased lipid peroxidation 3-fold ($P < 0.05$). A time–course study revealed that Adriamycin caused a progressive increase in peroxidation for as long as 90 min. It was also observed that Adriamycin-stimulated peroxidation was opti-

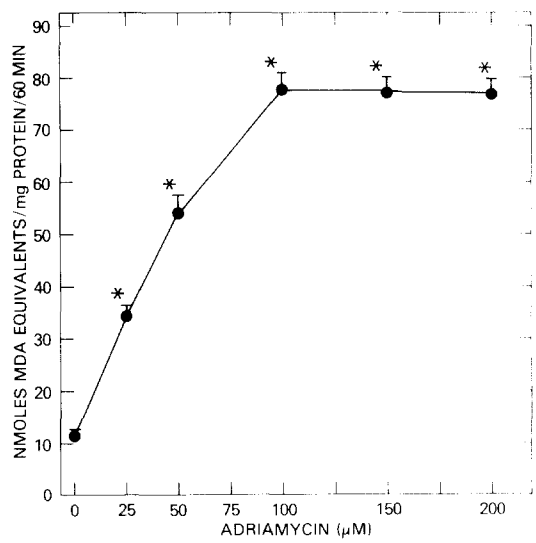

Fig. 1. Concentration-dependent stimulation of mouse kidney microsomal lipid peroxidation by Adriamycin. Microsomes were incubated for 60 min with an NADPH-generating system and various concentrations of Adriamycin. Data are expressed as nanomoles of malonaldehyde equivalents/mg protein/60 min (mean ± SD for three samples). Values significantly different from control values as determined by Student's *t*-test are shown by an asterisk ($P < 0.05$).

mal at 1 mg of microsomal protein/ml and with NADPH at 2.0 to 2.5 mM, and that heat-inactivated kidney microsomes did not peroxidize beyond zero-time values (data not presented). Under optimized incubation conditions, the maximum enhancement of mouse kidney microsomal peroxidation by Adriamycin approached 10-fold.

The extent of peroxidation in the presence of Adriamycin appeared to be maximal at 20–25 and 80–90 nmoles of malonaldehyde/mg protein for rat and mouse kidney microsomes respectively. The apparent resistance of rat kidney microsomes to lipid peroxidation could not be attributed to a higher content of the membrane antioxidant, α-tocopherol, because it was found that rat and mouse kidney microsomal α-tocopherol levels were equivalent (0.16 ± 0.01 and 0.14 ± 0.01 μg α-tocopherol/mg protein respectively). It is possible that substantial differences in the polyunsaturated fatty acid composition of mouse and rat kidney microsomes could have contributed to the differential susceptibility to peroxidation.

Inhibition of Adriamycin-stimulated lipid peroxidation by reactive oxygen scavengers and chelating agents. We next evaluated the involvement of reactive oxygen species in the stimulation of kidney microsomal lipid peroxidation by Adriamycin (Table 4). In these experiments NADPH was used in place of the NADPH-generating system, and the effects of specific scavengers of superoxide and hydroxyl radical were studied. The enzyme superoxide dismutase (EC 1.15.1.1) caused a significant decrease (>50%) in both endogenous and Adriamycin-enhanced peroxidation. Neither heat-inactivated superoxide dismutase nor intact nor heat-denatured bovine serum albumin caused any inhibition of peroxidation (intact bovine serum albumin slightly enhanced endogenous peroxidation).

The hydroxyl radical scavenger, 1,3-dimethylurea [50], diminished kidney microsomal peroxidation by more than 90% in either the absence or the presence of Adriamycin, strongly implicating an important role for hydroxyl free radical in the peroxidation process. Hydroxyl radical was most likely generated from the reaction of hydrogen peroxide (the dismutation product of superoxide) with trace amounts of endogenous ferrous iron ions (Fenton reaction) [51]. To verify this mechanism, we examined the effect of chelators of transition metal ions on the peroxidation reactions. Both EDTA and DETPAC were strongly inhibitory to endogenous and Adriamycin-enhanced lipid peroxidation (Table 4), most likely by preventing ferrous iron (or other reduced metal ions) from catalyzing the generation of hydroxyl radical.

The effect of catalase (EC 1.11.1.6) was not as clear-cut. Paradoxically, endogenous NADPH-dependent mouse kidney microsomal lipid peroxidation was increased to 850% by catalase (10^{-5} M), and Adriamycin-stimulated peroxidation was augmented to 150%. These results were unexpected because we have reported previously that catalase inhibits both liver microsomal [19] and liver mitochondrial lipid peroxidation [20]. Heat-inactivated catalase (10^{-5} M, immersed in a boiling water bath for 10 min) did not increase peroxidation (3.6 ± 0.4 nmoles MDA/mg protein/60 min), and the catalase-stimulated peroxidation could be blocked by DETPAC (10^{-5} M) and 1,3-dimethylurea (10^{-2} M) yielding 5.9 ± 0.3 and 4.7 ± 0.2 nmoles MDA/mg protein/60 min respectively.

Finally, the sulfhydryl-reactive reagent, *p*-chloro-

Table 4. Inhibition of mouse kidney microsomal lipid peroxidation by scavengers of oxy-radicals, chelators of metal ions and a sulfhydryl-reacting agent*

Additions to incubations† (concn)	Microsomal lipid peroxidation (nmoles MDA equivalents/mg protein/60 min)	
	Endogenous	Adriamycin-stimulated (100 μM)
None	4.8 ± 0.4‡	38 ± 6
Superoxide dismutase (6×10^{-5} M)	2.1 ± 0.2§	23 ± 1§
Superoxide dismutase (10^{-4} M)	1.2 ± 0.5§	19 ± 1§
BSA (3 mg/ml)	7.5 ± 0.3§	39 ± 4
Catalase (10^{-5} M)	41 ± 2§	59 ± 4§
1,3-Dimethylurea (10^{-2} M)	0.3 ± 0.2§	2.4 ± 0.2§
EDTA (10^{-5} M)	0.2 ± 0.1§	2.3 ± 0.4§
DETPAC (10^{-5} M)	0.0 ± 0.0§	1.2 ± 0.4§
p-CMB (5×10^{-4} M)	1.1 ± 0.5§	1.9 ± 0.1§

* Microsomes were incubated for 60 min with NADPH at 2.5 mM in the absence or presence of Adriamycin (100 μM), and lipid peroxidation was measured by the thiobarbituric acid method.

† Inhibitors were added to incubation mixtures just prior to NADPH. BSA was added to control for any nonspecific protein-related effect of superoxide dismutase and catalase. Heat-inactivated (boiling water bath for 1 min) superoxide dismutase (10^{-4} M) did not inhibit lipid peroxidation; heat-inactivated catalase (10^{-5} M, boiling water bath for 10 min) did not stimulate peroxidation.

‡ Values are expressed as the mean ± SD, N = 3–6.

§ Significantly different from values without added inhibitors, $P < 0.05$.

mercuribenzenesulfonic acid (*p*-CMB), potently prevented endogenous and Adriamycin-stimulated peroxidation, presumably by inhibiting the oxidoreductive enzymes that were obligatory for oxygen activation (Table 4).

Further enhancement of Adriamycin-stimulated lipid peroxidation by exogenous ferric and ferrous iron. The addition of exogenous ferrous sulfate (2–20 μM) increased NADPH-supported endogenous kidney microsomal lipid peroxidation 8-fold in a concentration-dependent manner. Adriamycin-enhanced lipid peroxidation was doubled by ferrous iron at an optimal concentration of 10 μM (Fig. 2). With ferric chloride in the range of 10–100 μM, endogenous peroxidation was increased 3-fold and Adriamycin-stimulated peroxidation 2-fold (Fig. 2). In the absence of NADPH, only a small amount of peroxidation was measured in the presence of either ferrous or ferric iron, with or without Adriamycin, indicating the obligatory requirement for a source of reducing equivalents. It is clear from the data in Fig. 2 that NADPH-dependent, ferrous or ferric iron-promoted lipid peroxidation was increased by Adriamycin at each of the iron concentrations that were studied.

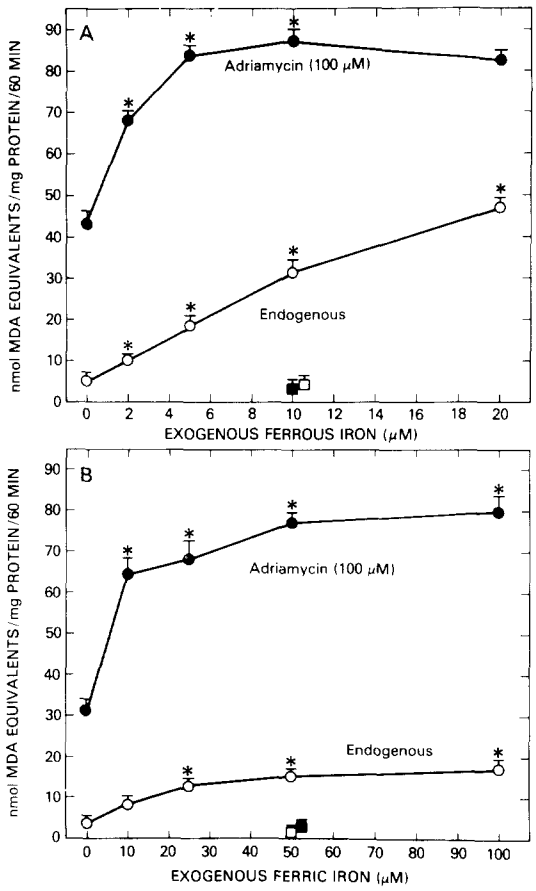

Fig. 2. Effects of exogenous ferrous (A) and ferric (B) iron ions on endogenous (○) and Adriamycin-stimulated (●) kidney microsomal lipid peroxidation. Values in the absence and presence of Adriamycin but without NADPH are shown by (□, ■). Data are expressed and were statistically analyzed as in the preceding figure. Values significantly different from control values are shown by an asterisk ($P < 0.05$).

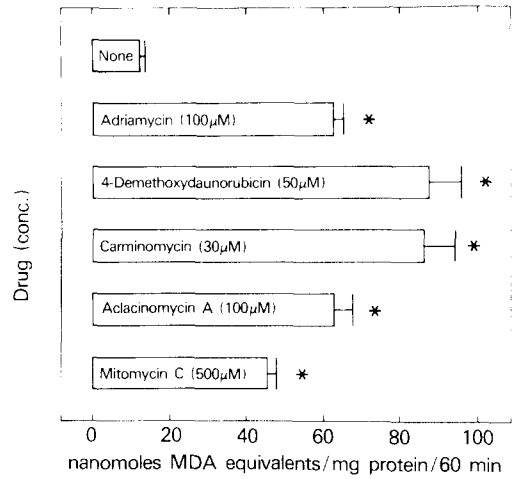

Fig. 3. Comparison of several quinone-containing anticancer agents for their ability to enhance NADPH-dependent (NADPH-generating system) mouse kidney microsomal lipid peroxidation. The drugs were added to incubation mixtures in a range of concentrations; however, only the concentrations that caused maximal enhancement of peroxidation are shown. Values (mean ± SD) significantly different from controls are shown by an asterisk, $P < 0.05$, $N = 3$–6.

Stimulation of kidney microsomal peroxidation by other anticancer drugs. Several other quinone-containing anthracyclines (4-demethoxydaunorubicin, carminomycin, and aclacinomycin A) and mitomycin C were capable of increasing kidney microsomal NADPH-dependent lipid peroxidation several-fold (Fig. 3). Among these drugs, carminomycin was conspicuous in that at 30 μM it was more potent than Adriamycin at 100 μM. Mitomycin C, which has been reported to be nephrotoxic in mice [13], dogs and rhesus monkeys [14] and humans [15], increased peroxidation 4-fold, albeit at a high drug concentration. Thus, Adriamycin was not unique in its ability to stimulate lipid peroxidation in kidney microsomes.

Mitochondrial NADH-dependent lipid peroxidation. Adriamycin-enhanced membrane lipid peroxidation was not limited to kidney microsomal fractions. The unsaturated membrane lipids of purified mouse and rat kidney mitochondria were susceptible to greatly increased NADH-dependent peroxidation in the presence of Adriamycin (Table 5). Mouse kidney mitochondrial lipid peroxidation was stimulated more than 12-fold by Adriamycin at 50 μM. The enhancement of mitochondrial lipid peroxidation by Adriamycin may result from the interaction of the drug with mitochondrial NADH-dehydrogenase (EC 1.6.99.3) [26] which can reduce Adriamycin to the free radical intermediate. Such an interaction has been demonstrated previously to result in greatly increased oxyradical production [30, 52, 53] and to stimulate lipid peroxidation in

Table 5. Enhancement by Adriamycin of mouse and rat kidney mitochondrial NADH-dependent lipid peroxidation*

Species	Additions	Lipid peroxidation (nmoles MDA equivalents/mg protein/60 min)
Mouse	None	2.2 ± 0.3†
	Adriamycin (50 μM)	28.4 ± 1.8‡
	Adriamycin (100 μM)	30.2 ± 0.9‡
Rat	None	3.2 ± 0.4
	Adriamycin (50 μM)	14.7 ± 1.2‡
	Adriamycin (100 μM)	16.2 ± 1.7‡

* Mitochondria were incubated at 1.0 mg protein/ml in the presence of NADH (2.5 mM) in KCl–Tris buffer, pH 7.4, for 60 min in the absence and presence of Adriamycin. Reactions were started by adding NADH, and lipid peroxidation was measured by the thiobarbituric acid method.

† Values are expressed as the mean ± SD for N = 4.

‡ Significantly greater than control values at $P < 0.001$.

mitochondria isolated from liver [20]. As noted above in the comparison of peroxidation in rat and mouse kidney microsomal fractions (Table 1), kidney mitochondria from mice were much more susceptible to membrane lipid peroxidation than were mitochondria isolated from rat kidney.

DISCUSSION

Following acute treatment with Adriamycin or daunorubicin, susceptible species develop a nephrotic syndrome characterized by proteinuria, hyperlipemia, peripheral edema and glomerular damage which leads to chronic glomerulonephritis [4, 54] and eventually kidney failure [8]. Morphological examination of Adriamycin-damaged rat or rabbit kidneys reveals extensive vacuolar destruction of juxtamedullary glomeruli with focal dilation of cisternae of the endoplasmic reticulum and marked invagination of the plasma membrane [8]. There is also cortical and medullary tubular dilation and degeneration, thickening of the basement membrane in Bowman's capsule and prominent interstitial fibrosis in advanced lesions [7, 55]. It is clear that many of the Adriamycin-caused morphological alterations in kidney, observed at the light microscopic and ultrastructural levels, are associated with extensive cellular membrane damage.

The biochemical mechanisms by which Adriamycin causes nephrotoxicity have not been defined clearly. Bristow *et al.* [4] have suggested that Adriamycin nephrotoxicity was mediated by the release of vasoactive histamine and catecholamines, and Bertani *et al.* [56] concluded that loss of glomerular fixed polyanion charges proceeded ultrastructural changes and proteinuria in Adriamycin-induced glomerulonephropathy, but these proposed mechanisms of anthracycline renal toxicity have not been investigated further. In a recent published study [57], it was observed that Adriamycin treatment raised lipid peroxide (malonaldehyde) levels 2-fold in mouse heart, liver and kidney 4 days after Adriamycin administration. Fajardo *et al.* [7] observed the frequent presence of fine lipofuscin pigment granules in the cytoplasm of renal proximal tubular cells in rabbits treated with Adriamycin; lipofuscin is an accumulation product of membrane lipid peroxidation [58]. The report that oxygen free radicals resulting from the action of xanthine oxidase caused lipid peroxidation which led to renal failure [35] also indicates that membrane lipid peroxidation can be associated with biochemical insults in kidney *in vivo*. It is also relevant that immune complex-mediated acute glomerular injury and kidney endothelial cell damage may be related to superoxide and hydrogen peroxide production by activated neutrophils [59].

Several previous studies have demonstrated that lipid peroxidation occurs in heart tissue *in vivo* following the administration of Adriamycin [17, 60], and greatly stimulated oxyradical-mediated lipid peroxidation occurs in isolated subcellular fractions from various tissues incubated *in vitro* with Adriamycin and appropriate pyridine nucleotide cofactors [18, 19, 53]. Considerable evidence suggests that, in microsomal or mitochondrial *in vitro* model systems, Adriamycin augments membrane lipid peroxidation as a consequence of enzymatic activation of the drug to the semiquinone free radical followed by the efficient generation of oxyradicals. We have reported previously that Adriamycin-enhanced microsomal and mitochondrial membrane lipid peroxidation could be potently inhibited by the antioxidants α-tocopherol and reduced glutathione as well as the reactive oxygen scavengers superoxide dismutase, catalase and 1,3-dimethylurea [19, 20, 22]. We therefore postulated that Adriamycin activation and oxyradical generation in kidney could cause oxidant stress in that organ which would manifest as membrane-damaging lipid peroxidation. Indeed, in the kidney microsome model system, this biochemical mechanism of toxicity appears to function as outlined.

In kidney microsomes, the activation of Adriamycin to a free radical intermediate is most likely catalyzed by the membrane-bound flavoproteins NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase [61], based on the capability of either NADPH or NADH to support Adriamycin-enhanced peroxidation. Pederson *et al.* [62] have

demonstrated the microsomal enzyme, NADPH–cytochrome P-450 reductase, to be intimately involved in NADPH-dependent lipid peroxidation in liver microsomes. NADH-dependent peroxidation of liver microsomal lipids appears to involve electron transfer through NADH–cytochrome b_5 reductase [63]. It is likely that enzyme-mediated peroxidation in kidney microsomes is at least qualitatively similar to that occurring in liver microsomes.

In the stepwise reduction of molecular oxygen, the sequence of partially reduced oxygen species is: superoxide, hydrogen peroxide, hydroxyl radical and, finally, water [64, 65] with the formation of the perhydroxyl radical from superoxide at acidic pH ($pK_a = 4.8$) [29]. Results presented here indicate that a similar oxyradical cascade mechanism of Adriamycin-enhanced lipid peroxidation occurs in microsomes from rodent kidney. The inhibition of kidney peroxidation by superoxide dismutase strongly implicates a role for superoxide as the initial product of oxygen reduction. Likewise, the diminution of peroxidation by 1,3-dimethylurea points toward the participation of hydroxyl radical in the peroxidation mechanism. Hydroxyl free radical is the most reactive oxygen species [66], and Fong *et al.* [67] have suggested hydroxyl radical to be a potent initiator of lipid peroxidation *in vitro*.

When we probed the kidney microsomal peroxidation reactions for hydrogen peroxide involvement by adding catalase, surprisingly we found that catalase enhanced the rate of lipid peroxidation. We observed previously that catalase significantly decreases endogenous and Adriamycin-stimulated peroxidation in liver microsomes [17] and mitochondria [18]. The addition of bovine serum albumin had no marked effect on endogenous or Adriamycin-augmented peroxidation in kidney microsomes, suggesting that the stimulation of peroxidation by catalase was not due to a nonspecific protein effect. Further experiments indicated that the enhancement of peroxidation by catalase could be blocked by the inclusion of DETPAC or 1,3-dimethylurea or be prevented by prior heat-inactivation of the catalase. At this time we have no solid explanation for this anomalous catalase effect other than to suggest that the enzyme was converted to an intermediate form with peroxidase-like activity [68]. Such a catalase intermediate, under the conditions of assay might augment the peroxidation of membrane unsaturated lipids by catalyzing lipid alkoxy radical formation from fatty acid hydroperoxides. This interesting and potentially toxicologically relevant observation is currently under further investigation.

The inhibition of endogenous and Adriamycin-stimulated lipid peroxidation by the metal ion chelators EDTA and DETPAC implies that metal ions, most likely iron or copper, have an important role in the peroxidation process. In addition, the enhancement of Adriamycin-stimulated peroxidation by exogenous ferric and ferrous iron salts supports this contention. The effects of the combination of Adriamycin with ferric or ferrous iron ions on kidney microsomal peroxidation was not strictly additive. At low concentrations of iron (2–4 μM Fe^{2+}; 10–20 μM Fe^{3+}) a pronounced synergy was observed with Adriamycin. These results may indicate that Adriamycin-complexed iron was involved, as has been suggested previously in other peroxidation systems [69, 70]. In our experiments, the iron was added to the reaction mixtures separately and subsequent to Adriamycin; however, the possibility exists that an iron–Adriamycin complex of unknown stoichiometry was formed. At all ferrous or ferric iron concentrations studied, Adriamycin greatly increased lipid peroxidation (compare for example the effects of Adriamycin at 5 μM Fe^{2+} or at 50 μM Fe^{3+}). What is unclear is whether iron salts (or an iron–Adriamycin complex) enhanced the initiation and propagation of lipid peroxidation by increasing hydroxyl radical generation, accelerated the decomposition of fatty acid hydroperoxides, or simply increased the proportion of malonaldehyde that was produced as a by-product of peroxidation. It may also be premature to conclude that exogenous and endogenous iron ions function in the same manner, even in the absence of Adriamycin. Further experiments are called for to understand the role or roles for metal ions in Adriamycin-enhanced microsomal lipid peroxidation. The situation *in vivo* is undoubtedly much more complex.

The differential effects of the anthracycline analogs on kidney microsomal peroxidation may be attributed to their relative ability to interact with the flavin moiety of NADPH–cytochrome P-450 reductase [53], to differences in their oxidation–reduction potentials or to the rates of reaction of the free radical intermediates with molecular oxygen. Presumably, in this model system, the oxyradical cascade beyond superoxide would be the same in the presence of each agent. It is also possible that the ability of these drugs to bind iron ions could influence their ability to stimulate lipid peroxidation. Careful mechanistic studies with anthracycline analogs are required to characterize these factors.

In conclusion, it is clear from the work presented here that Adriamycin and other anthracycline antitumor drugs are capable of greatly enhancing the peroxidation of membrane unsaturated lipids *in vitro* in isolated subcellular fractions from mouse and rat kidney by an oxyradical-mediated process. These observations warrant the consideration that oxyradical-mediated peroxidative damage to cellular membrane structure and function may play a role in the pathogenesis of anthracycline nephrotoxicity.

Acknowledgements—The authors wish to thank Tanya Prather, Beth Singer and Katherine Brown for assistance in preparing this manuscript.

REFERENCES

1. L. Giroux, C. Smeesters, F. Boury, M. P. Faure and G. Jean, *Lab. Invest.* **50**, 190 (1984).
2. S. S. Donaldson, P. S. Moskowitz, E. L. Canty and L. F. Fajardo, *Int. J. Radiat. Oncol. Biol. Phys.* **6**, 851 (1980).
3. D. M. Young, *Cancer Chemother. Rep.* **6**, 159 (1975).
4. M. R. Bristow, W. A. Minobe, M. E. Billingham, J. B. Marmor, G. A. Johnson, B. M. Ishimoto, W. S. Sageman and J. R. Daniels, *Lab. Invest.* **45**, 157 (1981).
5. J. F. Van Vleet, L. A. Greenwood and V. J. Ferrans, *Am. J. vet. Res.* **40**, 1537 (1979).
6. J. F. Burke, Jr., J. F. Laucius, H. S. Brodovsky and R. Z. Soriano, *Archs Intern. Med.* **137**, 385 (1977).

7. L. F. Fajardo, J. R. Eltringham, J. R. Stewart and M. R. Klauber, *Lab. Invest.* **43**, 242 (1980).
8. J. F. Van Vleet and V. J. Ferrans, *Am. J. vet. Res.* **41**, 1462 (1980).
9. S. K. Carter, *J. natn. Cancer Inst.* **55**, 1265 (1975).
10. J. H. Doroshow, G. Y. Locker, I. Ifrim and C. E. Myers, *J. clin. Invest.* **68**, 1053 (1981).
11. C. E. Myers, W. P. McGuire and R. Young, *Cancer Treat. Rep.* **60**, 961 (1976).
12. R. A. Buroker, D. F. Johnson and H. Madissoo, *Drug chem. Toxic.* **4**, 383 (1981).
13. K. Kuroda, S. Teranishi and M. Akao, *Gann* **73**, 656 (1982).
14. F. S. Philips, H. S. Schwartz and S. S. Sternberg, *Cancer Res.* **20**, 1354 (1960).
15. K. Liu, A. Mittelman, E. E. Sproul and E. G. Elias, *Cancer, N.Y.* **28**, 1314 (1971).
16. R. D. Olson, J. S. MacDonald, C. J. Van Boxtel, R. C. Boerth, R. D. Harbison, A. E. Slonim, R. W. Freeman and J. A. Oates, *J. Pharmac. exp. Ther.* **215**, 450 (1980).
17. C. E. Myers, W. P. McGuire, R. H. Liss, J. Ifrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
18. J. Goodman and P. Hochstein, *Biochem. biophys. Res. Commun.* **77**, 797 (1977).
19. E. G. Mimnaugh, M. A. Trush and T. E. Gram, *Biochem. Pharmac.* **30**, 2797 (1981).
20. E. G. Mimnaugh, M. A. Trush, M. Bhatnagar and T. E. Gram, *Biochem. Pharmac.* **34**, 847 (1985).
21. E. G. Mimnaugh, K. A. Kennedy, M. A. Trush and B. K. Sinha, *Cancer Res.* **45**, 3296 (1985).
22. E. G. Mimnaugh, T. E. Gram and M. A. Trush, *J. Pharmac. exp. Ther.* **226**, 806 (1983).
23. S. Sato, M. Iwaizumi, K. Handa and Y. Tamura, *Gann* **68**, 603 (1977).
24. N. R. Bachur, S. L. Gordon and M. V. Gee, *Molec. Pharmac.* **13**, 901 (1977).
25. N. R. Bachur, M. V. Gee and R. D. Friedman, *Cancer. Res.* **42**, 1078 (1982).
26. J. H. Doroshow, *Cancer Res.* **43**, 4543 (1983).
27. K. Handa and S. Sato, *Gann* **66**, 43 (1975).
28. B. Kalyanaraman, E. Perez-Reyez and R. P. Mason, *Biochim. biophys. Acta* **630**, 119 (1980).
29. J. M. Gebicki and B. H. J. Bielski, *J. Am. chem. Soc.* **103**, 7020 (1981).
30. W. S. Thayer, *Chem. Biol. Interact.* **19**, 265 (1977).
31. T. Komiyama, T. Kikuchi and Y. Sugiura, *Biochem. Pharmac.* **31**, 3651 (1982).
32. J. S. Bus and J. E. Gibson, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), p. 125. Elsevier: North Holland, New York (1979).
33. R. O. Recknagel and E. A. Glende, Jr., in *Free Radicals in Molecular Biology and Pathology* (Ed. W. A. Pryor), Vol. 3, p. 97. Academic Press, New York (1973).
34. I. Pasquali-Ronchetti, A. Bini, B. Botti, G. DeAlojsio, C. Fornieri and V. Vannini, *Lab Invest.* **42**, 457 (1980).
35. M. S. Paller, J. R. Hoidal and T. F. Ferris, *J. clin. Invest.* **74**, 1156 (1984).
36. T. E. Gram, C. L. Litterst and E. G. Mimnaugh, *Drug Metab. Dispos.* **2**, 254 (1974).
37. W. C. Schneider and G. H. Hogeboom, *J. biol. Chem.* **183**, 123 (1950).
38. F. Bernheim, M. L. Bernheim and K. M. Wilber, *J. biol. Chem.* **174**, 257 (1948).
39. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
40. C. H. Williams, Jr. and H. Kamin, *J. biol. Chem.* **237**, 587 (1962).
41. K. Mihara and R. Sato, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 102. Academic Press, New York (1978).
42. S. L. Taylor, M. P. Lamden and A. L. Tappel, *Lipids* **11**, 530 (1976).
43. G. W. Snedecor, *Statistical Methods*, p. 45. Iowa State University Press, Ames, IA (1956).
44. M. Yonaha and Y. Ohbayashi, *Res. Commun. Chem. Path. Pharmac.* **30**, 113 (1980).
45. S. V. Shah, F. C. Cruz, W. H. Baricos and V. Beck, *Kidney Int.* **23**, 691 (1983).
46. C. L. Litterst, E. G. Mimnaugh, R. L. Reagan and T. E. Gram, *Drug Metab. Dispos.* **3**, 259 (1975).
47. D. J. Kornbrust and R. D. Mavis, *Lipids* **35**, 315 (1980).
48. A. Ottolenghi, *Archs Biochem. Biophys.* **79**, 355 (1959).
49. S. F. Wong, B. Halliwell, R. Richmond and W. R. Skrorvoneck, *J. inorg. Biochem.* **14**, 127 (1981).
50. J. Tibaldi, J. Benjamin, F. S. Cabbat and R. E. Heikkila, *J. Pharmac. exp. Ther.* **211**, 415 (1979).
51. I. Fridovich, *Science* **201**, 875 (1978).
52. J. H. Doroshow, *Cancer Res.* **43**, 460 (1983).
53. E. D. Kharasch and R. F. Novak, *J. Pharmac. exp. Ther.* **226**, 500 (1983).
54. S. S. Sternberg, *Lab. Invest.* **23**, 39 (1970).
55. D. Deprez-DeCampeneere, R. Jaenke and A. Trouet, *Cancer Treat. Rep.* **66**, 395 (1982).
56. T. Bertani, A. Poggi, R. Pozzoni, F. Delaini, G. Sacchi, Y. Thoua, G. Mecca, G. Remuzzi and M. B. Donati, *Lab. Invest.* **46**, 16 (1982).
57. H. Tanizawa, Y. Sazuka and Y. Takino, *Chem. pharm. Bull., Tokyo* **31**, 1714 (1983).
58. A. L. Tappel, *Ann. N.Y. Acad. Sci.* **203**, 12 (1972).
59. R. Rehan, K. J. Johnson, R. C. Wiggins, R. G. Kunkel and P. A. Ward, *Lab. Invest.* **51**, 396 (1984).
60. L. H. Patterson, B. M. Gadecha and J. R. Brown, *Biochem. biophys. Res. Commun.* **110**, 399 (1983).
61. S. S. Pan, L. Pedersen and N. R. Bachur, *Molec. Pharmac.* **19**, 184 (1980).
62. T. C. Pederson, J. A. Buege and S. D. Aust., *J. biol. Chem.* **248**, 7134 (1973).
63. Y. Hirokata, A. Shigematsu and T. Omura, *J. Biochem., Tokyo* **83**, 431 (1978).
64. R. C. Bray, *Biochem. J.* **117**, 13P (1970).
65. B. Halliwell and J. M. C. Gutteridge, *Biochem. J.* **219**, 1 (1984).
66. G. Czapski, in *Methods in Enzymology* (Ed. L. Packer), Vol. 105, p. 209. Academic Press, New York (1984).
67. K. L. Fong, P. B. McCay, J. L. Poyer, B. B. Keele and H. Misra, *J. biol. Chem.* **248**, 7792 (1973).
68. B. Halliwell, in *Age Pigments* (Ed. R. S. Sohol), p. 1. Elsevier/North-Holland, New York (1981).
69. H. Muliawan, M. E. Schenlen and H. Kappus, *Biochem. Pharmac.* **31**, 3147 (1982).
70. K. Sugioka and M. Nakano, *Biochim. biophys. Acta* **713**, 333 (1982).