Biochemical Pharmacology, Vol. 40, No. 10, pp. 2343–2351, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

EFFECTS OF MANSONONES ON LIPID PEROXIDATION, P450 MONOOXYGENASE ACTIVITY, AND SUPEROXIDE ANION GENERATION BY RAT LIVER MICROSOMES*

SILVIA FERNANDEZ VILLAMIL,†‡ MARTA DUBIN,†§ CONRADO GALEFFI‖ and ANDRÉS O. M. STOPPANI†§¶

† Centro de Investigaciones Bioenergéticas, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121-Buenos Aires, Argentina; and ‖ Departamento di Chimica del Farmaco, Instituto Superiore di Sanita, Roma, Italia

(*Received* 29 *March* 1990; *accepted* 14 *June* 1990)

Abstract—Several structurally related *ortho*-naphthoquinones isolated from *Mansonia altissima* Chev (mansonones C, E and F) (a) inhibited NADPH-dependent, iron-catalyzed microsomal lipid peroxidation; (b) prevented NADPH-dependent cytochrome P450 destruction; (c) inhibited NADPH-supported aniline 4-hydroxylase activity; (d) inhibited Fe(III)ADP reduction by NADPH-supplemented microsomes; (e) stimulated superoxide anion generation by NADPH-supplemented microsomes; and (f) stimulated ascorbate oxidation. ESR investigation of ascorbate-reduced mansonone F demonstrated semiquinone formation. Mansonone C had a greater effect than mansonones E and F on NADPH-dependent lipid peroxidation, O_2^- production and ascorbate oxidation, whereas mansonone E was more effective than mansonones C and F on aniline 4-hydroxylase activity. Mansonones E and F did not inhibit hydroperoxide-dependent lipid peroxidation, cytochrome P450 destruction or microsomal aniline 4-hydroxylase activity. Mansonone C inhibited to a limited degree *tert*-butyl hydroperoxide-dependent lipid peroxidation, this inhibition being increased by NADPH. Mansonone A, a tetrahydro *ortho*-naphthoquinone derivative, was in all respects relatively less effective than mansonones C, E and F. It is postulated that mansonones C, E and F inhibited microsomal lipid peroxidation and cytochrome P450 catalyzed reactions by diverting reducing equivalents from NADPH to dioxygen, but mansonone C (including its reduced form) may also exert direct antioxidant activity.

The lipophilic *ortho*-naphthoquinones β-lapachone**, CG 8-935, CG 9-442 and CG 10-248 inhibit NADPH-dependent microsomal lipid peroxidation, prevent NADPH-dependent cytochrome P450 destruction, and inhibit several cytochrome P450 catalyzed reactions [1]. The same quinones stimulate NADPH oxidation, superoxide anion radical generation and Fe(III)ADP reduction by NADPH-supplemented microsomes, in accordance with Reactions 1–3 where Q, $QH^{\cdot}$ and QH_2 are the quinone, semiquinone and reduced

$$NADPH + H^+ + Q \rightarrow NADP^+ + QH_2 \quad (1)$$

$$QH_2 + Q \rightarrow 2\,QH^{\cdot} \quad (2)$$

$$QH^{\cdot} + O_2 \rightarrow Q + O_2^- + H^+ \quad (3)$$

quinone respectively. These observations support the hypothesis that lipophilic *o*-naphthoquinones inhibit microsomal lipid peroxidation and cytochrome P450-catalyzed reactions, by diverting reducing equivalents from NADPH to dioxygen [2, 3].

Mansonones (Fig. 1) are sesquiterpenoid *o*-naphthoquinones extracted from the heart-wood of *Mansonia altissima* Chev [4], a Sterculiacea from tropical West Africa. These compounds may be responsible for the irritative symptoms in workers handling Mansonia sawdust and also for the toxicity of the corresponding extracts [4]. In the present study, we examined mansonone effects on lipid peroxidation, cytochrome P450-catalyzed reactions, the production of oxygen radicals, and related processes in rat liver microsomes.

MATERIALS AND METHODS

Microsomal preparations. Microsomes were obtained from the livers of 20-hr fasted, male Wistar rats, 240–280 g, fed a Purine-like rat chow. Following

* This paper is dedicated to Professor G. B. Marini Bettolo on the occasion of his 75th birthday.

‡ Research Fellow, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

§ Career Investigator, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

¶ Corresponding author.

** Abbreviations and chemical terms: β-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; α-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[2,3*b*]-pyran-5,10-dione; CG 8-935, 3,4-dihydro-2-methyl-2-ethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; CG 9-442, 3,4-dihydro-2-methyl-2-phenyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; CG 10-248, 3,4-dihydro-2,2-dimethyl-9-chloro-2*H*-naphtho-[1,2*b*]pyran-5,6-dione; mansonone A, 5,6,7,8-tetrahydro-3,8-dimethyl-5-isopropyl-1,2-naphthoquinone; mansonone C, 3,8-dimethyl-5-isopropyl-1,2-naphthoquinone; mansonone E, 2,3-dihydro-3,6,9-trimethyl-naphtho[1,8-*bc*]pyran-7,8-dione; mansonone F, 3,6,9-trimethyl-naphtho[1,8-*bc*]pyran-7,8-dione; *t*-BuOOH, *tert*-butyl hydroperoxide; CuOOH, cumenyl hydroperoxide; DMFA, dimethyl formamide; SOD, superoxide dismutase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; NADPH-generating system, 0.55 mM $NADP^+$, 5.5mM $MgCl_2$, 5.5. mM G6P and G6PD (1.2 units/mL); MDA, malondialdehyde; DETAPAC, diethylenetriaminepentaacetic acid.

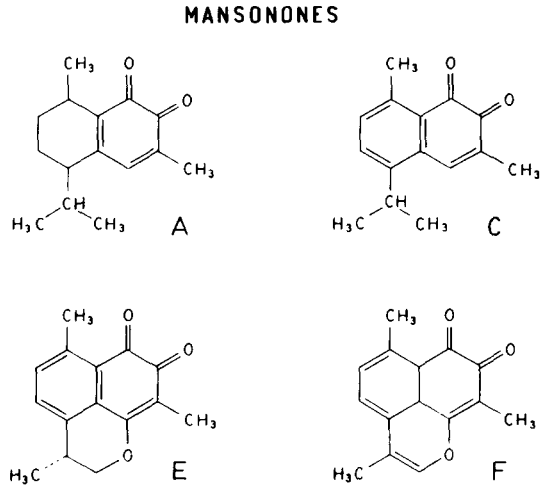

Fig. 1. Structures of mansonones A, C, E and F.

homogenization of the livers in Tris–KCl buffer (50 mM Tris–HCl, 150 mM KCl, pH 7.4) and centrifugation at 11,000 *g* (15 min), the supernatant was centrifuged for 60 min at 105,000 *g*. The microsomal pellet was washed twice with 150 mM KCl by centrifugation for 1 hr at 105,000 *g*, resuspended in 150 mM KCl, and either used immediately or stored in liquid nitrogen. Investigation of superoxide dismutase in the microsomal suspension was negative, whereas measurement of catalase activity yielded 9.0 milliunits/mg protein.

Assay of lipid peroxidation. For the assay of NADPH-dependent lipid peroxidation, the incubation mixture contained liver microsomes (1.5 mg protein/mL), the NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM G6P, 1.2 units/mL G6PD, 5.5 mM $MgCl_2$), 1.7 mM ADP, 0.1 mM $FeCl_3$, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4. The $FeCl_3$-ADP mixture was prepared separately before beginning the experiments. After thermal equilibration at 37°, the reaction was started by addition of G6PD. Aliquots (1.0 mL) were withdrawn at the times indicated and chilled to 0°, and MDA formation was measured. For the *t*-BuOOH-induced peroxidation, the incubation mixture contained 2.6 mM *t*-BuOOH, microsomes (1.5 mg protein/mL) and 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4. The mansonones were added dissolved in DMFA. Controls received the same volume of solvent whose concentration never exceeded 1% (v/v); DMFA failed to affect the rate of lipid peroxidation. The final volume of the incubation mixture was always 3.0 mL. Incubations were performed in a New Brunswick gyratory shaker, at 60 cycles/min and 37°, under air. Other experimental conditions were as described in Results.

Enzyme assays. Catalase activity was measured as described by Aebi [5]. Aniline 4-hydroxylase activity was measured by determining 4-aminophenol production, which was estimated colorimetrically by the indophenol reaction [6]. For the assay using NADPH as electron donor, the reaction mixture contained microsomes (1.7 mg protein/mL), 1.25 mM aniline, 6.25 mM $MgCl_2$, 2.5 mM G6P, G6PD (0.5 units/mL), 0.125 mM $NADP^+$, 0.1 M KH_2PO_4-Na_2HPO_4, pH 7.4, preflushed with O_2. Incubation was for 20 min at 37°. For the assay using CuOOH, the reaction mixture contained microsomes (2.0 mg protein/mL), 3.0 mM aniline, 0.15 mM CuOOH, 80 mM Tris–HCl buffer, pH 7.6. Incubation was for 30 min at 37°. NADPH–cytochrome P450 reductase activity was measured with NADPH as substrate and cytochrome *c* as artificial electron acceptor [7]. The reductase-catalyzed transfer of electrons to cytochrome *c* was measured spectrophotometrically at 550 minus 540 nm. NADH–cytochrome b_5 reductase was estimated by the rate of reduction of potassium ferricyanide followed spectrophotometrically at 420 nm [8]. Spectrophotometric measurements were performed at 30° using the Aminco DW 2a™ UV/VIS spectrophotometer. Other experimental conditions are described in Results. Enzyme activities are expressed in nanomoles of substrate transformed per minute per milligram of protein.

Reduction of Fe(III)ADP *complex.* The reduction was monitored at 37° in 0.15 M Tris–HCl buffer, pH 7.4, in the presence of microsomes [0.17 mg protein/mL, 0.10 mM NADPH, 5 mM G6P, G6PD (0.5 units/mL), 64 μM Fe(III)ADP (molar ratio Fe(III)/ADP = 1:100)]. During reduction under air, the reoxidation of ferrous iron was inhibited by 0.3 mM 2,2′-dipyridyl and the formation rate of the stable ferrous-$(bipyridyl)_3$ complex was measured at 510 nm [9]. The absorbance change was calibrated by the addition of excess dithionite at the end of each experiment and was taken as internal standard. Spectrophotometric measurements were performed using the Perkin–Elmer 5505 UV/VIS spectrophotometer.

Determination of O_2^- *and* H_2O_2 *generation.* Production of O_2^- was determined by the cytochrome *c* [10] and the adrenochrome [11] assays. With the former method, the reaction mixture contained microsomes (0.13 mg protein/mL), 130 mM KCl, 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4, 9.6 μM acetylated cytochrome *c* and either NADPH-generating system or 0.16 mM NADPH; total volume was 3.0 mL. The reaction was started either with G6PD or NADPH, and the reaction rate was measured by the increase of absorption at 550 minus 540 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$). With the adrenochrome method, the reaction mixture contained microsomes (0.27 mg protein/mL), 1.0 mM epinephrine, KCl, phosphate buffer and NADPH-generating system as above; total volume was 3.0 mL. The reaction rate was measured by the increase of absorption at 480 minus 575 nm ($\varepsilon = 2.96\ mM^{-1}\ cm^{-1}$). Production of H_2O_2 was determined by the microperoxidase assay [12] by measuring the absorption change at 419 minus 407 nm ($\varepsilon = 78\ mM^{-1}\ cm^{-1}$). The reaction mixture consisted of microsomes (62 μg protein/mL), 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4, 130 mM KCl, 3 μM microperoxidase (MP-11) and NADPH-generating system; total volume was 3.0 mL. Absorption measurements were performed with the Aminco DW 2a™ UV/VIS spectrophotometer, at 30°. SOD addition was always 7 units/mL.

Analytical methods. MDA and protein content of microsomal membranes was determined as in Refs. 13 and 14 respectively. Mansonones did not interfere with the MDA assay. Cytochrome P450 content of microsomes was estimated as described by Omura and Sato [15], and cytochrome b_5 was estimated from its redox spectrum of NADH-reduced versus oxidized cytochrome [16]. Spectra were recorded using the Aminco DW-2^{TM} UV/VIS spectrophotometer. Oxygen uptake was measured with a Gilson Oxygraph, model 5/6, using the Clark electrode.

Electron spin resonance (ESR). Measurements were performed with a Bruker ER 200 tt x-band ESR spectrometer applied with a TE_{102} cavity, at room temperature (28°). The experimental conditions were: microwave power, 21 mW; modulation amplitude, 0.20 G; time constant, 0.5 sec; and scan rate, 6 G/min. A 3-mL sample of quinone solution in 130 mM KCl, 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4, was flushed with a stream of nitrogen; then 15 mM sodium ascorbate was added and the ESR spectrum was recorded.

Chemicals. Mansonones A, C, E and F were prepared as described [4]. G6P, G6PD (from baker's yeast), SOD (from dog erythrocytes), microperoxidase (MP-11), cytochrome *c* (Type VI), $NADP^+$, NADPH, DETAPAC, ADP (Grade IV), sodium ascorbate, *t*-BuOOH, CuOOH, DMFA, thiobarbituric acid, aniline, 4-aminophenol, epinephrine, sodium dithionite, bovine serum albumin (A6003) and Trizma were purchased from the Sigma Chemical Co., St. Louis, MO. 2,2′-Dipyridyl was purchased from C. Erba, Milano. Carbon monoxide was purchased from Matheson, East Rutherford, NJ. Phenol, ferric chloride, and DMFA were purchased from Mallinckrodt Chemical Works, New York, NY. Cytochrome *c* was acetylated using acetic anhydride in the presence of sodium-acetate. Other reagents were as in Ref. 1.

Expression of results. The values presented are the average of duplicate samples; the experimental values deviated from the mean by less than 5%. When quadruplicate samples were measured, the values presented are the mean ± SE. Statistical analysis was performed using Student's *t*-test for paired values.

RESULTS AND DISCUSSION

Table 1 shows the effects of mansonones on MDA production by liver microsomes. Lipid peroxidation was initiated either by NADPH or *t*-BuOOH, and the incubation time was 60 min. The results presented indicate that at a 5.0 μM concentration, mansonones C and E inhibited the NADPH-induced lipid peroxidation by 96 and 71%, respectively, but mansonones F and A were less effective inhibitors. At a 1.0 μM concentration only mansonone C produced a relatively minor inhibition of the NADPH-dependent lipid peroxidation, thus setting the lower limit for the activity of the quinones. In contrast to these results, mansonones failed to affect the *t*-BuOOH-dependent lipid peroxidation. Table 2 shows, however, that at relatively early incubation times, mansonone C inhibited the *t*-BuOOH-dependent lipid peroxidation. NADPH increased the mansonone effect, in close agreement with previous observations of Talcott *et al.* [17] and Dubin *et al.* [1] using menadione and CG *ortho*-naphthoquinones respectively. The results in Table 2 suggest inhibition of lipid peroxidation propagation by mansonone C, especially by its reduced form.

The hepatic microsomal monooxygenase system consists of cytochrome P450 and NADPH–cytochrome P450 reductase [18] and loss of microsomal cytochrome P450 has been associated with both NADPH- and hydroperoxide-induced lipid peroxidation [19–22]. Accordingly, mansonone inhibition of lipid peroxidation prevented cytochrome P450 destruction in microsomes incubated with the NADPH-generating system. Figure 2 illustrates the results of a typical experiment, using mansonones C and E as inhibitors. It is to be seen that after incubation, cytochromes P450 and b_5 decreased by 83 and 18%, respectively, these variations being nullified by the assayed quinones. Cytochrome P450 loss did not represent conversion into cytochrome P420 as was to be expected from the observations of Lame and Segall [21]. Table 3 summarizes the results obtained when using the NADPH-generating system to produce lipid peroxidation and hemoprotein destruction. The data presented show that mansonones C, E and F prevented the NADPH-dependent destruction of cytochrome P450. Measurement of cytochrome b_5 in the same microsomes revealed the expected [23] diminution of this hemoprotein. Mansonones C and E prevented cytochrome b_5 destruction to some extent, but failed to affect the NADPH–cytochrome P450 and NADH–cytochrome b_5 reductase activities (experimental data omitted).

Differences in the time–course of cytochome P450 destruction produced by NADPH and CuOOH afforded an alternative procedure for determining the site of action of the active mansonones [22]. Figure 3 shows that when using NADPH to initiate lipid peroxidation, the time–course of hemoprotein loss involved two phases, namely, a slow initial one of about 6 min, and a faster terminal one. In contrast to these results, the CuOOH-induced hemoprotein destruction followed monotonic kinetics. In close agreement with the results in Fig. 2 and Table 3, mansonone C completely inhibited the NADPH-induced but not the CuOOH-dependent cytochrome P450 loss (Fig. 3). The latter results are apparently at variance with those reported in Table 2. Nevertheless, in the absence of NADPH, the effect of mansonone C was limited (Table 2), and the lipid peroxidation products formed could be sufficient to destroy cytochrome P450.

In cytochrome P450-catalyzed reactions, electrons donated from NADPH are transferred to cytochrome P450 through NADPH–cytochrome P450 reductase [24]. Considering that the latter enzyme catalyzes quinone redox-cycling, it was assumed that this reaction may cause diversion of electrons away from cytochrome P450, thereby inhibiting cytochrome P450-dependent reactions. To test this hypothesis, the effects of mansonones, at fixed concentrations, on aniline 4-hydroxylase activity, were determined. Table 4 shows that mansonones

Table 1. Effects of mansonones on lipid peroxidation by liver microsomes incubated with the NADPH-generating system or *t*-BuOOH

Mansonone	Concentration (μM)	MDA-equivalents (nmol/mg protein)	
		NADPH-generating system	*t*-BuOOH
None		60.5	5.6
A	1	59.5 (2)	
	5	53.1 (12)	5.5
C	1	55.8 (8)	
	5	2.6 (96)	5.4
E	1	59.3 (2)	
	5	17.5 (71)	5.7
F	1	62.6 (−3)	
	5	46.6 (23)	5.6

Microsomes were incubated with the reaction mixture (composition as described in Materials and Methods); incubation time, 60 min. Percent inhibition of lipid peroxidation is given in parentheses.

Table 2. Effect of mansonone C on *t*-BuOOH-dependent microsomal lipid peroxidation

Mansonone C (μM)	Time of incubation (min)	MDA production (nmol equivalent/mg protein)	
		−NADPH	+NADPH
0	10	4.49	3.83
5		3.28 (27)	1.55 (59)
0	20	5.47	4.97
5		4.60 (16)	2.89 (42)
0	30	6.28	6.01
5		5.79 (8)	3.68 (39)

The reaction mixture consisted of microsomes (1.4 mg protein/mL), 2.6 mM *t*-BuOOH, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4, pH 7.4; 5 μM mansonone C and 0.5 mM NADPH were added as indicated above. Other experimental conditions were as described in Materials and Methods. Values represent the average of duplicate samples; zero-time values were subtracted. Percent inhibition of lipid peroxidation is given in parentheses.

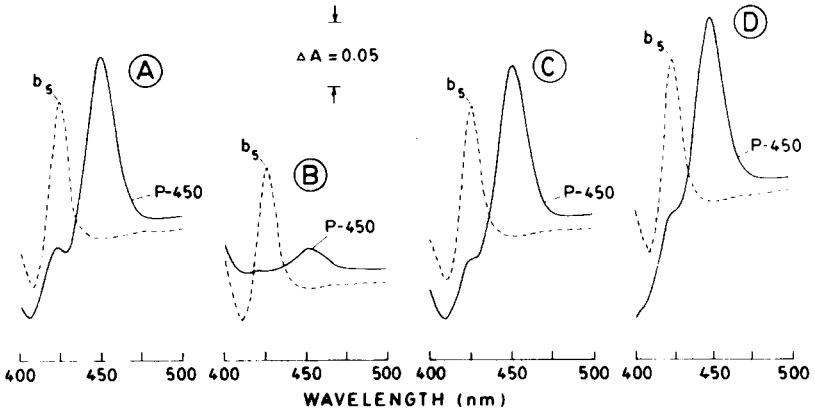

Fig. 2. Effects of mansonones on cytochromes P450 and b_5 destruction after incubation with the NADPH-generating system. Samples contained microsomes (1.5 mg protein/mL), 130 mM KCl, 23 mM KH_2PO_4-Na_2HPO_4, pH 7.4, and the following additions: sample A, none; sample B, 0.1 mM Fe(III)ADP (molar ratio 1:17), NADPH-generating system and 5.0 μL DMFA; sample C, same as B, except for DMFA which was replaced by 5.0 μL of mansonone C solution (final concentration 5.0 μM); sample D, same as B, except for DMFA which was replaced by 5 μL of mansonone E solution; total volume 6.0 mL. After a 1-hr incubation at 37°, the suspensions were centrifuged at 105,000 *g* for 30 min, the microsomes were suspended in 6.0 mL of 0.1 M phosphate buffer, pH 7.4, and cytochromes P450 and b_5 were measured in duplicate 3.0-mL samples. Other conditions were as described in Materials and Methods. Results of a typical experiment are presented.

Table 3. Effects of mansonones on cytochrome P450 and cytochrome b_5 loss after microsome incubation with the NADPH-generating system

Mansonone (5 μM)	Hemoprotein loss after incubation (% of control)		P450/b_5 molar ratio
	P450	b_5	
None	83	17	0.33
C	2	11	1.84
E	7	6	1.59
F	40	17	1.17

Microsomes were incubated with the NADPH-generating system as described in the legend of Fig. 2. Samples contained microsomes (1.5 mg protein/mL), 130 mM KCl, 23 mM KH_2PO_4-Na_2HPO_4, pH 7.4, 0.1 mM Fe(III)ADP (molar ratio 1:17), NADPH-generating system, and mansonone as indicated above. After a 1-hr incubation at 37°, the suspensions were centrifuged at 105,000 *g* for 30 min, the microsomes were suspended in 6.0 mL phosphate buffer, pH 7.4, and cytochromes were measured in duplicate 3.0-mL samples. Other conditions were as described in Materials and Methods. Control microsomes were incubated without NADPH-generating system; cytochrome content (nmol/mg protein): 0.58 (P450) and 0.36 (b_5). Values represent the average of duplicate measurements.

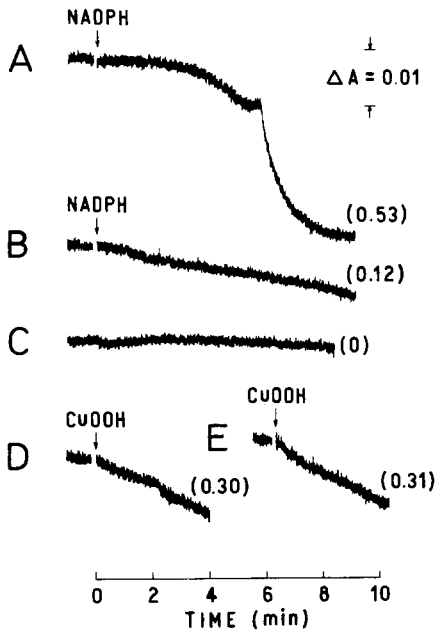

Fig. 3. Effect of mansonone C on the time-course of NADPH- and CuOOH-induced losses of microsomal cytochrome P450. A 5.0-mL microsomal suspension (1.0 mg protein/mL) in 0.1 M KH_2PO_4-Na_2HPO_4, pH 7.4, containing 150 μM NADH was divided equally between two cuvettes and placed in the sample and reference cuvette chambers of the Aminco DW 2a™ UV/VIS spectrophotometer, at 30°. Additions were as follows: samples A, C and D, 5.0 μL DMFA: samples A and B, 0.1 mM Fe(III)ADP; samples B and E, 5.0 μL mansonone C (final concentration, 5.0 μM). Destruction of cytochrome P450 was initiated by adding either NADPH (final concentration 600 μM, samples A and B) or CuOOH (final concentration 2.6 mM, samples D and E). C is a control sample. Absorption at 418 nm was measured for the time indicated on the abscissa. Values in parentheses represent the total loss of cytochrome P450 (in nmol/mL) and are the average of duplicate measurements. Other conditions were as described in Materials and Methods.

inhibited the NADPH-induced aniline 4-hydroxylase but did not inhibit the CuOOH-induced activity [25], NADPH–cytochrome P450 reductase or cytochrome b_5 reductase, thus indicating the site-specificity of their action. The greatest inhibition was produced by mansonone E and the least by mansonone A, intermediate values being obtained for mansonones C and F. Inhibition of cytochrome P450 reactions may imply inhibitor binding which can be monitored by spectral changes in the Soret absorption of cytochrome P450 [26, 27]. Under standard experimental conditions [26, 27] and using 25 μM mansonone C, no spectral changes, however, were observed (data omitted). Apparently, inhibition of cytochrome P450-dependent electron transfer and inhibition of lipid peroxidation by mansonone C obeyed somewhat different mechanisms, although in both cases electron diversion by the quinone at the NADPH–cytochrome P450 reductase level played the leading role.

In the presence of NADPH, liver microsomes rapidly reduce the Fe(III)ADP complex [9]. This reduction may be essential for initiating lipid peroxidation and, therefore, we examined the effect of mansonone C on Fe(III)ADP reduction. The results obtained are presented in Fig. 4 and may be described as follows. In the system comprised of microsomes, NADPH, Fe(III)ADP and quinone, two different reactions occurred simultaneously: (a) Fe(III) reduction to Fe(II); and (b) mansonone C reduction to semiquinone (or quinol). These reactions competed for NADPH-donated electrons and were mutually exclusive. Therefore, by diverting electrons, the mansonone inhibited Fe(III) reduction. On the other hand, oxidation of the semiquinone (or quinol) by oxygen generates $O_2^{\cdot-}$, which in turn reduces Fe(III) to Fe(II) [28]. Since (a) O_2 reduction by the semiquinone could not account for all the electrons following the quinone pathway, and (b) Fe(III) reduction could not account for all the $O_2^{\cdot-}$ formed by the quinone redox-cycling, inhibition of Fe(III) reduction became apparent (Fig. 4, upper line). However, when the quinone concentration increased, $O_2^{\cdot-}$ production increased as

Table 4. Effects of mansonones on microsomal aniline-4-hydroxylase activity

Mansonone	Concentration (μM)	Enzyme activities (nmol/min/mg protein)			
		Aniline 4-hydroxylase		NADPH–cytochrome P450 reductase	Cytochrome b_5 reductase
		"NADPH"	CuOOH		
A	5	0.67 (8)		85	3.11
	10	0.22 (17)	0.41		
C	5	0.59 (19)		106	3.12
	10	0.44 (30)	0.43		
E	5	0.41 (44)		95	4.0
	10	0.22 (65)	0.41		
F	5	0.58 (20)		82	3.5
	10	0.34 (46)	0.43		
None*		0.73		81	3.3
None†		0.63	0.41		

Experimental conditions were as described in Materials and Methods. Microsomes were incubated with the NADPH-generating system ("NADPH") or with CuOOH as indicated above. Mansonones were added in 5 or 10 μL of DMFA solution, the control samples containing the same volume of solvent. Values for aniline 4-hydroxylase activity represents the mean of four measurements; SE < 0.02, in all cases; inhibition (%) of enzyme activity is given in parentheses.

* Control for the 5 μM mansonone samples.

† Control for the 10 μM mansonone samples.

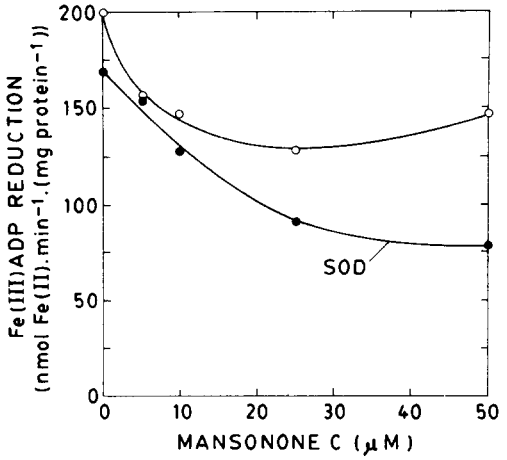

Fig. 4. Effect of mansonone C on the reduction rate of the Fe(III)ADP complex. Experimental conditions were as described in Materials and Methods. Fe(III) reduction was monitored continuously by the rate of formation of the Fe(II)-dipyridil complex, at 510 nm. The points represent the mean of duplicate measurements. SOD, 7 units/mL.

well, thus explaining the biphasic shape of the upper line in Fig. 4. Addition of SOD eliminated O_2^- and, accordingly, the effect of SOD depended on the amount of O_2^- formed. At the lowest mansonone C concentration, O_2^- production was small and, therefore, the SOD effect was hardly detectable. The reverse occurred at the higher quinone concentrations (Fig. 4, SOD line). The foregoing provides important evidence for mansonone C inhibition of lipid peroxidation initiation.

The antioxidant activity of a given quinone may bear a relation to its activity as a substrate for microsomal "quinone reductase" or, in other words, to the possibility of quinone redox-cycling, which can be demonstrated by O_2^- generation. Results in Table 5 support this hypothesis since mansonone C was about 4-fold more effective than mansonone A as O_2^- generator. O_2^- production was demonstrated by cytochrome *c* reduction and the effect of SOD. Similar results were obtained using the adrenochrome method (experimental data omitted). Flavoprotein-catalyzed quinone redox-cycling may involve two-electron transfer reactions [29]. However, with the assayed mansonones, the H_2O_2 production rate was always less than 3% of the corresponding O_2^- value (experimental data omitted).

The redox-cycling capability of lipophilic *o*-naphthoquinone depends on the relative rates of Reactions 1–3, particularly on the quinone reduction and the semiquinone oxidation rates [30]. To test these kinetic possibilities, the reduction rates of mansonones A, C and E were measured anaerobically, as described in Fig. 5. After quinone reduction, the reaction mixtures were aerated and quinol oxidation was determined. The results obtained may be described as follows: (a) the mansonone A reduction rate was negligible and, obviously, no quinol oxidation could be measured, (b) mansonones C and E were reduced rapidly, and (c) oxygen admission produced partial (mansonone C) or almost total (mansonone E) quinol oxidation. The latter difference may be explained by the faster rate of Reaction 1 with mansonone C (Fig. 5), which under steady-state conditions would determine a higher $[QH_2]/[Q]$ ratio. This assumption fits in well with results in Table 5 since according to the rate equation $dO_2^-/dt = k_3[QH_2][O_2]$, O_2^- production rate was linearly related to the quinol concentration [30].

Quinones are reduced by ascorbate to semiquinone [31] and one electron transfer from the semiquinone to dioxygen yields the superoxide anion radical [32]. Oxygen uptake provided then a measure of free-radical production [33, 34], and ESR measurements of the mansonone C–ascorbate mixture

Table 5. Effects of mansonones on superoxide anion formation by liver microsomes

Mansonone (5 μM)	Superoxide anion production (nmol/min/mg protein) −SOD	+SOD	Inhibition (%)
None	3.52 ± 0.48	1.56 ± 0.61	66
A	5.19 ± 0.65*	2.73 ± 0.30†	48
C	23.9 ± 1.0	3.19 ± 0.16†	87
E	15.0 ± 1.9	6.47 ± 0.94†	57
F	11.4 ± 0.8	4.77 ± 0.31†	58

$O_2^{\cdot}$ production was measured with the cytochrome *c* method, as described in Materials and Methods. Values are means ± SE (N = 4).

*† Analysis of variance: * P, non-significant (mansonone A against control sample); and † $P < 0.01$ (+SOD against −SOD samples).

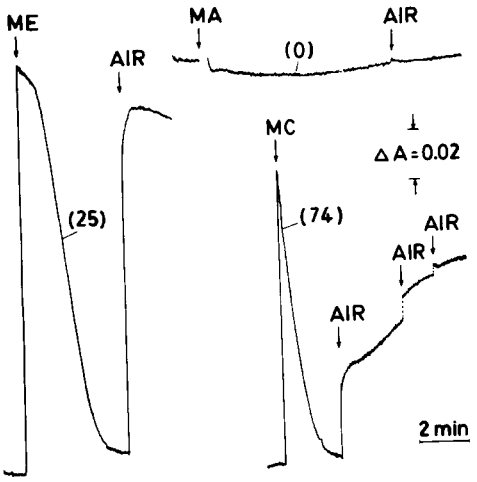

Fig. 5. Mansonone redox-cycling with NADPH as electron donor. The reaction mixture contained microsomes (0.45 mg protein/mL), 50 μM mansonone A, C or E, 0.6 mM NADPH, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4, pH 7.4; total volume, 3.0 mL. Samples were incubated in a stoppered septum cap cuvette pre-flushed with argon for 10 min. The reaction was started by adding the quinone solution pre-flushed with argon. MA, MC and ME = mansonones A, C and E respectively. Absorption variation was measured at 432 nm (MA), 446 nm (MC) or 450 nm (ME), using the Aminco DW 2a™ UV/VIS spectrophotometer, at 30°. ε ($mM^{-1}\,cm^{-1}$): 0.96 (MA), 2.14 (MC) and 3.34 (ME). The numbers in parentheses represent the mansonone reduction rate (nmol/min/mL). Where indicated by the arrows, air was introduced into the reaction mixture. Other experimental conditions were as described in Materials and Methods.

revealed the semiquinone signal (Fig. 6; tracing B, corresponds to the semidehydroascorbate radical [34]. Table 6 shows the different capabilities of mansonones for their action on ascorbate oxidation. In close agreement with mansonone effects on $O_2^{\cdot}$ production (Table 5), mansonones C, E and F enhanced oxygen uptake, but mansonone A did not. Mansonone E was, however, somewhat less active than F, at variance with its effect on $O_2^{\cdot}$ production. Anaerobic reduction of mansonones A and C by ascorbate (Fig. 7) confirmed the results obtained with the microsomal system (Fig. 5).

The results here described extend previous observations with other lipophilic *o*-naphthoquinones, such as β-lapachone and related CG compounds [1]. These observations support the view [1, 2] that quinone reduction by NADPH–cytochrome P450 reductase and semiquinone (or quinol) oxidation by dioxygen divert electrons, thus preventing lipid peroxidation and cytochrome P450-dependent reactions. However, mansonone C exerted direct antioxidant activity (Table 2) in good agreement with the observations of Talcott *et al.* [17], Kostyuk [27] and Dubin *et al.* [1] using other quinones. Hence, it should be concluded that the inhibition of lipid peroxidation by quinones may be a complex process in which, depending on the quinone structure, inhibition of the propagation process may contribute to the overall quinone effect.

Comparison of the effects of the mansonones assayed led to some structure–activity correlations. Lipid peroxidation inhibition, $O_2^{\cdot}$ production stimulation and quinone anaerobic reduction were of a much lesser extent or were not observed with mansonone A, the tetrahydronaphthoquinone derivative. Accordingly, the naphthoquinone benzene ring should be critical for mansonone capability for redox-cycling and oxygen radical production. The pyrane ring, which contributes to the oxaphenalene structure of mansonones E and F, exerted a lesser, though varied influence on the activities of the mansonones. Thus, mansonone C was more effective than E and F on NADPH-dependent lipid peroxidation (Table 1), $O_2^{\cdot}$ production (Table 5), and ascorbate oxidation (Table 6), but E was more active than C on aniline 4-hydroxylase activity (Table 4). As regards mansonones E and F, the former was more active on lipid peroxidation (Table 1) and $O_2^{\cdot}$ production (Table 5), whereas the latter was more effective on ascorbate oxidation (Table 6). Structure–activity studies with several naphthoquinones [17] have demonstrated the importance of substitutents on the naphthoquinone nucleus that may serve to stabilize forms capable of high antioxidant activity. Similar studies on mansonones are needed to elucidate their specific behavior as inhibitors of lipid peroxidation and microsomal electron transfer.

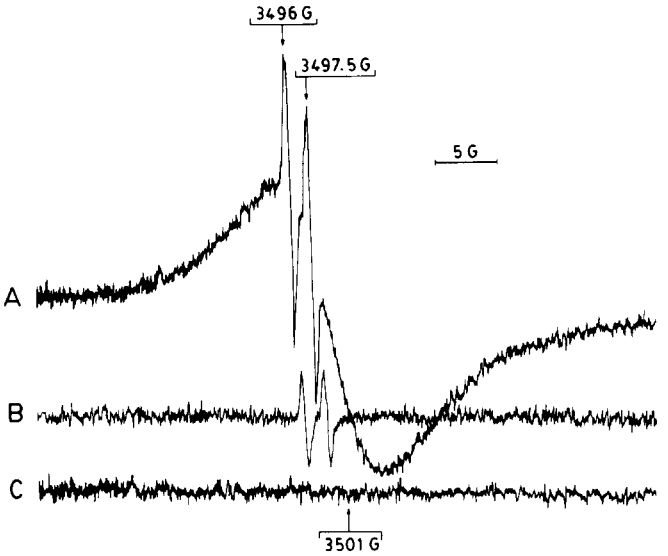

Fig. 6. ESR-signals of ascorbate-reduced mansonone C. The reaction mixture contained 90 mM ascorbate, 1.5 mM quinone, 0.1 M Tris–HCl, pH 7.6. It was flushed with nitrogen for 5 min, placed in the spectrometer cell and left for 10–15 min before recording the spectrum. Other experimental conditions were as described in Materials and Methods. Key: A, sample containing quinone plus ascorbate; B, same, without quinone; and C, same, without ascorbate. A typical experiment is shown.

Table 6. Effects of mansonones on ascorbate oxidation

Mansonone	Concentration (μM)	Oxygen uptake (nmol/min/mL)
A	50	6
	250	22
C	50	109
	250	185
E	50	12
	250	45
F	50	36
	250	138
None		0

Oxygen uptake was measured polarographically, at 30°. The reaction mixture contained 5.5 mM ascorbate, 1.0 mM DETAPAC, 100 mM Tris–HCl buffer, pH 7.5, and mansonone as stated above. Other conditions were as described in Materials and Methods. Values are the averages of duplicate samples.

Finally, the production of oxyradicals by mansonones C, E and F may help to explain the toxic effects observed in workers handling Mansonia sawdust and the corresponding extracts [4].

Acknowledgements—This work was aided by grants from the Scientific Office of the Organization of American States, Consiglio Nazionale delle Ricerche (Italia), Universidad de Buenos Aires and CEDIQUIFA (Buenos Aires). We are grateful to Professor G. B. Marini Bettolo (Centro di Studio per la Chimica dei Recettori e Delle Molecole Biologicamente Attive, Universitā Cattolica del Sacro Cuore, Roma, Italia) for his interest in this work and to Doctors L. J. Perisinotti, M. A. Brusa (Mar del Plata) and A. Distefano (Buenos Aires) for the ESR measurements. L. T. Viñas and M. G. Gutierrez lent able technical assistance.

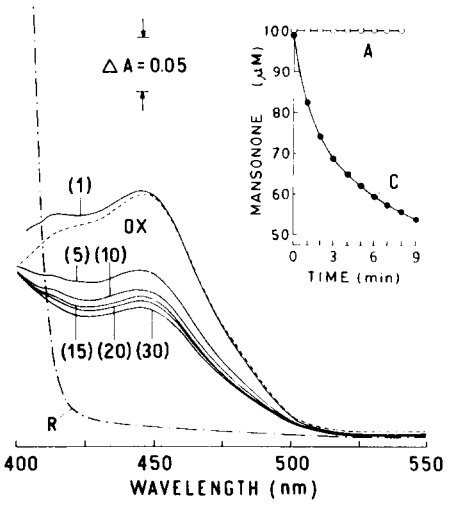

Fig. 7. Mansonone C redox-cycling with ascorbate as electron donor. The reaction mixture contained 100 μM mansonone C, 5.0 mM sodium-ascorbate, 0.1 M Tris–HCl buffer, pH 7.5, and 1.0 mM DETAPAC; total volume, 3.0 mL. Incubation was under argon, as described in the legend of Fig. 5. The reaction was started by adding the quinone, and the anaerobic spectra were recorded at the times (min) indicated by the figures in parentheses. The reaction mixture was then aerated, and spectrum OX was recorded. R = dithionite-reduced mansonone spectrum. *Insert*: Anaerobic reduction rate of mansonones A and C. Values were calculated from the absorption decrease at 432 nm (A) or 446 nm (C). Other conditions were as described above and in the legend of Fig. 5.

REFERENCES

1. Dubin M. Fernandez Villamil SH and Stoppani AOM, Inhibition of microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions by β-lapachone and related naphthoquinones. *Biochem Pharmacol* **39**: 1151–1160, 1990.
2. Wills ED, Effects of vitamin K and naphthoquinones on lipid peroxide formation and oxidative demethylation by liver microsomes. *Biochem Pharmacol* **21**: 1879–1883, 1972.
3. Högberg J, Orrenius S and O'Brien PJ, Further studies on lipid-peroxide formation in isolated hepatocytes. *Eur J Biochem* **59**: 449–455, 1975.
4. Galeffi C, Casinovi CG, Miranda Delle Monache E and Marini-Bettolo GB, Chinoni sesquiterpenici della *Mansonia altissima* Chev.—Nota II. *Ann Ist Super Sanita* **4**: 305–316, 1968.
5. Aebi H, Catalase *in vitro*. *Methods Enzymol* **105**: 121–126, 1984.
6. Lake BG, Determination of aniline 4-hydroxylase. In: *Biochemical Toxicology* (Eds. Snell K and Mullock B), pp. 206–207. IRL Press, Oxford, 1987.
7. Lake BG, Determination of NADPH–cytochrome *c* (P450) reductase. In: *Biochemical Toxicology* (Eds. Snell K and Mullock B), pp. 200–201. IRL Press, Oxford, 1987.
8. Rogers MJ and Strittmatter P, The interaction of NADH–cytochrome b_5 reductase and cytochrome b_5 bound to egg lecithin liposomes. *J Biol Chem* **250**: 5713–5718, 1975.
9. Végh M, Marton A and Horváth I, Reduction of Fe(III)ADP complex by liver microsomes. *Biochim Biophys Acta* **964**: 146–150, 1988.
10. McCord JM and Fridovich I, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244**: 6049–6055, 1969.
11. Misra HP and Fridovich I, The univalent reduction of oxygen by reduced flavins and quinones. *J Biol Chem* **247**: 188–192, 1972.
12. Paget TA, Fry M and Lloyd D, Effects of inhibitors on the oxygen kinetics of *Nippostrongylus brasiliensis*. *Mol Biochem Parasitol* **22**: 125–133, 1987.
13. Placer ZA, Cushman L and Johnson BC, Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem* **16**: 359–364, 1966.
14. Gornall AG, Bardawill CJ and David MM, Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177**: 751–766, 1949.
15. Omura T and Sato R, Isolation of cytochromes P-450 and P-420. *Methods Enzymol* **10**: 556–561, 1967.
16. Lake BG, Determination of cytochrome b_5. In: *Biochemical Toxicology* (Eds. Snell K and Mullock B), p. 194. IRL Press, Oxford, 1987.
17. Talcott RE, Smith MT and Giannini DD, Inhibition of microsomal lipid peroxidation by naphthoquinones: Structure–activity relationships and possible mechanisms of action. *Arch Biochem Biophys* **241**: 88–94, 1985.
18. Taniguchi H, Imai Y and Sato R, Protein–protein and lipid interactions in a reconstituted cytochrome P-450 dependent microsomal monooxygenase. *Biochemistry* **26**: 7084–7090, 1987.
19. Nerland DE, Iba MM and Mannering GJ, Use of linoleic acid hydroperoxide in the determination of absolute spectra of membrane-bound cytochrome P-450. *Mol Pharmacol* **19**: 162–167, 1981.
20. Minotti G, Borrello S, Palombini G and Galeotti T, Cytochrome P-450 deficiency and resistance to *t*-butyl hydroperoxide of hepatoma microsomal lipid peroxidation. *Biochim Biophys Acta* **876**: 220–225, 1986.
21. Lame MW and Segall HJ, *In vitro* effects of *trans*-4-hydroxy-2-alkenals on mouse liver cytochrome P-450. *Chem Biol Interact* **62**: 59–74, 1987.
22. Iba MM and Mannering GJ, NADPH- and linoleic acid hydroperoxide-induced lipid peroxidation and destruction of cytochrome P-450 in hepatic microsomes. *Biochem Pharmacol* **36**: 1447–1455, 1987.
23. Buege JA and Aust SD, Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302–310, 1978.
24. Murray M, Mechanisms of the inhibition of cytochrome P-450-mediated drug oxidation by therapeutic agents. *Drug Metab Rev* **18**: 55–81, 1987.
25. Karuzina II, Varenitsa AI and Archakov AI, Comparative inhibitor analysis of the hydroxylation of aniline by cytochrome P-450 in NADPH-, cumene hydroperoxide-, and H_2O_2-dependent systems. *Biokhimiya* **48**: 1788–1793, 1983.
26. Lake BG, Determination of the spectral interaction of xenobiotics with cytochrome P-450. In: *Biochemical Toxicology* (Eds. Snell K and Mullock B), pp. 197–200. IRL Press, Oxford, 1987.
27. Kostyuk VA, Inhibition of microsomal oxidation by *o*-benzoquinone derivatives. *Biokhimiya* **52**: 355–358, 1987.
28. Borg DC and Schaich KM, Iron and iron-derived radicals. In: *Oxygen Radicals and Tissue Injury* (Ed. Halliwell B), pp. 20–26. The Upjohn Company, Bethesda, MD, 1988.
29. Cēnas NK, Rakauskienē GA and Kulys JJ, One- and two-electron reduction of quinones by glutathione reductase. *Biochim Biophys Acta* **973**: 399–404, 1989.
30. Boveris A, Docampo R, Turrens JF and Stoppani AOM, Effect of β-lapachone on superoxide anion and hydrogen peroxide production in *Trypanosoma cruzi*. *Biochem J* **175**: 431–439, 1978.
31. Gascoyne PRC, Determination of quinone-reductase activities in whole cells and purified enzymes from free radical decay kinetics. *Acta Biochim Biophys Hung* **22**: 251–261, 1987.
32. Smith MT, Evans CG, Thor H and Orrenius S, Quinone-induced oxidative injury to cells and tissues. In: *Oxidative Stress* (Ed. Sies H), pp. 91–113. Academic Press, London, 1985.
33. Powis G, Metabolism and reactions of quinoid anticancer agents. *Pharmacol Ther* **35**: 57–162, 1987.
34. Rao DNR, Harman L, Motten A, Schreiber J and Mason RP, Generation of radical anions of nitrofurantoin, misonidazole, and metronidazole by ascorbate. *Arch Biochem Biophys* **255**: 419–427, 1987.