Biochemical Pharmacology, Vol. 33, No. 21, pp. 3419–3423, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

EFFECT OF NITROHETEROCYCLIC DRUGS ON LIPID PEROXIDATION AND GLUTATHIONE CONTENT IN RAT LIVER EXTRACTS

MARTA DUBIN, SILVIA G. GOIJMAN and ANDRÉS O. M. STOPPANI*
Centro de Investigaciones Bioenergéticas, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

(*Received* 21 *September* 1983; *accepted* 9 *March* 1984)

Abstract—Incubation of rat liver cell-free extracts with an NADPH-generating system and with nifurtimox or benznidazole (two nitroheterocyclic drugs used in the treatment of Chagas' disease) produced oxidation of reduced glutathione (GSH) and increased lipid peroxidation, as shown by the generation of thiobarbituric-acid-reacting intermediates. Nifurtimox and benznidazole inhibited GSSG-reductase, but not GSH-peroxidase, the former inhibition contributing to GSH depletion. In every case, nifurtimox was more effective than benznidazole. Addition of GSH or free-radical scavengers (catalase, superoxide dismutase, mannitol, sodium benzoate or L-histidine) prevented the effect of nifurtimox on lipid peroxidation reactions. These results support the assumption [M. Dubin, S. N. J. Moreno, E. E. Martino, R. Docampo and A. O. M. Dubin, *Biochem. Pharmac.* **32**, 483 (1983)] that, in the rat liver, GSH exerts a protective action against oxygen radicals generated by the nitroheterocyclic drugs.

Nifurtimox† and benznidazole, two nitroheterocyclic drugs currently used for the treatment of Chagas' disease, generate superoxide anion, hydrogen peroxide and hydroxyl radicals, when added to liver microsomes in the presence of NADPH [1–3]. In good agreement with these reactions *in vitro*, treatment of rats with pharmacological doses of nifurtimox induces a time- and dose-dependent depletion of liver glutathione and extra release of GSSG and GSH into bile [4]. The extra release of GSSG demonstrates nifurtimox-dependent H_2O_2 generation in the liver cells, since (a) the rate of the GSH-peroxidase reaction (Reaction 1) is a function of the H_2O_2 intracellular concentration [5] and (b) nifurtimox does not inhibit GSSG-reductase *in vivo* [4] (Reaction 2). Incubation of rat liver microsomes with nitroheterocyclic

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (1)$$

$$GSSG + NADPH + H^+ \rightleftarrows 2GSH + NADP^+ \quad (2)$$

drugs causes lipid peroxidation, as shown by the increase of conjugated dienes in the microsomal lipid extract and the increased rate of malondialdehyde generation [1, 2]. However, in *in vivo* experiments, nifurtimox treatment does not increase the rate of peroxidation of liver lipids [4]. This negative result leads one to assume that, in intact liver cells, protective mechanisms, involving in all probability GSH-peroxidase [5], prevent lipoperoxidation by reducing oxygen radical concentration to ineffective levels. To test this hypothesis, in the present study we examined the effects of nifurtimox and benznidazole on GSH and GSSG levels, lipid peroxidation, GSH-peroxidase and GSSG-reductase activities, in liver cell-free extracts. The results described here show a correlation between GSH depletion and rate of malondialdehyde generation, nifurtimox being in all cases more effective than benznidazole.

MATERIALS AND METHODS

Liver extracts. Wistar male rats (200–250 g) from the Instituto de Química Biológica were used in the experiments. Animals were fed "Nutrimento" (Purina-like) rat chow; the protein content of the diet was 23.4% and included all the essential amino acids. After decapitation (without anesthesia), the liver was rapidly removed, weighed, washed and suspended in ice-cold KCl–Tris buffer (150 mM KCl–50 mM Tris–HCl, pH 7.4), in the proportion of 3 ml/g liver. Homogenization was performed in a Potter tissue grinder with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 15 min at 4°, and the supernatant fraction was used in the experiments.

Reagents. $NADP^+$, NADPH, *tert*-butyl hydroperoxide, GSSG, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase (from bakers yeast; Type V), glutathione reductase (from yeast, Type III), TBA, DTNB, catalase (from bovine liver; 30,000 units/mg protein), superoxide dismutase (from bovine liver; 3,000 units/mg protein), mannitol, sodium benzoate and L-histidine were purchased from the Sigma Chemical Co., St. Louis, MO; malondialdehyde bis-(dimethyl acetal) was purchased from the Aldrich Chemical Co, Milwaukee,

* To whom all correspondence should be addressed.

† Abbreviations: nifurtimox, 3-methyl-4-(5′nitrofurfuryldene-amino)-tetrahydro-4*H*-, 4-thiazone-1, 1′-dioxide; benznidazole, *N*-benzyl-2-nitro-1-imidazole acetamide; GSSG, oxidized glutathione; GSH, reduced glutathione; GSSG-reductase, glutathione reductase (EC 1.6.4.2; reduced NAD(P):oxidized glutathione oxidoreductase); GSH-peroxidase, glutathione peroxidase (EC 1.11.1.9; glutathione:hydrogen-peroxide oxidoreductase); TBA, thiobarbituric acid; DTNB, 5,5′-dithiobis-(nitrobenzoic acid); and TCA, trichloroacetic acid.

WI. Nifurtimox and benznidazole were supplied by Bayer A. G. Leverkusen, German Federal Republic, and Hoffmann-La Roche, Basel, Switzerland, respectively. Other reagents were of analytical grade.

Incubation. In each experiment, duplicate samples were incubated at 37°, in a New Brunswick Gyratory model 45 water bath. The standard reaction mixture contained 0.20 ml of liver extract (50–60 mg protein), 0.1 M phosphate buffer, pH 7.4, and NADPH-generating system (0.5 mM $NADP^+$, 5 mM D-glucose-6-phosphate, 0.25 mM $MgCl_2$ and 3.5 units D-glucose-6-phosphate dehydrogenase); total volume was 2.5 ml. During incubation, samples were shaken at a rate of 40 rpm.

Analytical methods. Products of lipid peroxidation were measured with the TBA reaction [6]. One milliliter of incubation mixture was cooled at 4°, and 1.0 ml of 4 N HCl and 1.0 ml of 0.8% (w/v) TBA were added. The supernatant absorbance was measured at 532 nm against a reagent blank, containing, when necessary, nifurtimox or benznidazole at the concentration used in the incubated sample. A Perkin Elmer Spectrophotometer, model 550S, was used for these measurements. Malondialdehyde concentration was calculated from a standard curve obtained with malondialdehyde bis(dimethyl acetal). The GSH content in the incubation mixture was measured using the method of Ellman [7] as modified by Sedlack and Lindsay [8]. One milliliter of mixture was precipitated with 1 ml of 10% (w/v) TCA and, after centrifugation, to 1 ml of supernatant fraction was added 4.5 ml of 0.1 mM DTNB in 0.1 M phosphate buffer, pH 8.0. Absorbance was measured at 412 nm against a reagent blank containing, when necessary, nifurtimox or benznidazole (as above). GSH concentration was calculated using $\varepsilon = 13.5\ mM^{-1} \cdot cm^{-1}$. No difference was detected when the resulting values were compared with those obtained by the enzymatic method [9]. GSSG was measured by following NADPH oxidation in the presence of GSSH-reductase. The reaction mixture contained 50 mM phosphate buffer ($K_2HPO_4 \cdot KH_2PO_4$, pH 7.0), 0.5 mM NADPH, 0.1 unit/ml of yeast GSSG-reductase and 50–100 μl incubation mixture, in a final volume of 3 ml. Total glutathione, i.e. GSH plus 2GSSG, was determined using GSSG-reductase and DTNB, as described in Refs. 10 and 11. The reaction mixture contained 50 mM phosphate buffer (as above), 0.1 unit/ml GSSG-reductase, 0.5 mM NADPH, 33 μM DTNB and 50–100 μl incubation mixture, in a final volume of 3.0 ml. Absorbance was measured at 412 nm. After measuring the reaction initial velocity, a sample of GSSG of known concentration was added as a standard. Results are given as GSH, and the GSH content was calculated by subtracting GSSG measured values (as described above) from total glutathione values. Protein was determined by the biuret method [12].

Determination of enzyme activities. The activities of GSSG-reductase and GSH-peroxidase were measured at 30° GSSG-reductase was measured essentially as described in Ref. 13. The reaction mixture contained 50 mM phosphate buffer (as above), 0.5 to 1.0 mM GSSG, 0.5 mM NADPH and 25–50 μl of incubation mixture; final volume was 3 ml. GSH-peroxidase was measured by a modification of the coupled assay procedure [13, 14]. The reaction mixture contained 50 mM phosphate buffer (as above), 1 mM GSH, 0.5 mM NADPH, 0.1 unit/ml yeast GSSG-reductase, 0.5 mM *tert*-butyl hydroperoxide and 25–50 μl incubation mixture; final volume was 3 ml. In all cases NADPH oxidation was followed by measuring the decrease of absorption at 340 nm.

Statistical analysis. This was performed using Student's *t*-test. The values presented are the average of three to six independent experiments.

RESULTS

Figure 1 shows the effects of nifurtimox and benznidazole on free GSH concentration in liver extracts. Initial values, related either to 1 mg of soluble protein (Fig. 1) or to 1 g of liver tissue (6.3 to 9.3 μmoles GSH), agreed well with those reported previously [4, 9, 15, 16]. Nifurtimox addition pro-

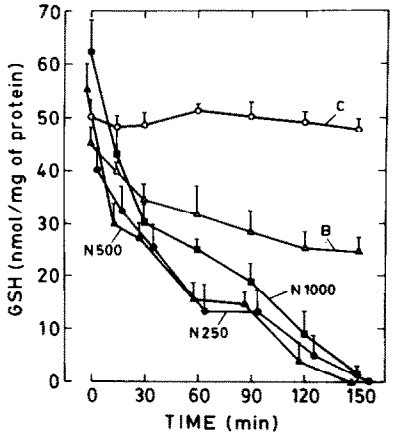

Fig. 1. Effects of nifurtimox (N) and benznidazole (B) on GSH content in liver extracts supplemented with an NADPH-generating system. Experimental conditions were as described in the text. C indicates the control sample; the numbers represent nifurtimox concentration (μM); the benznidazole concentration was 2.0 mM. Each point represents the average of six determinations ± S.E. P for 0-time samples, > 0.05.

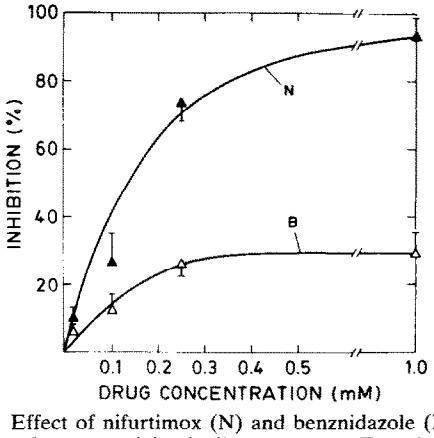

Fig. 2. Effect of nifurtimox (N) and benznidazole (B) on GSSG-reductase activity in liver extracts. Experimental conditions were as described under Materials and Methods Each point represents mean value ± S.E.

Table 1. Effects of nifurtimox and benznidazole on GSH oxidation in liver extracts*

Drug (mM)	GSH (nmoles/mg protein)	GSSG (nmoles/mg protein)	
		Calculated	Determined
None	56.2 ± 12.2†	10.9	12.0 ± 3.4
Nifurtimox (0.25)	26.3 ± 7.1	25.9	26.3 ± 9.2
(1.0)	24.6 ± 2.4	26.7	24.4 ± 7.8
None	50.4 ± 9.5	13.8	14.7 ± 6.2
Benznidazole (2.0)	35.2 ± 6.3	21.4	23.3 ± 4.1

* Experimental conditions were as described in the legend of Fig. 1; incubation time, 1 hr. Total GSH (GSH + 2GSSG): 78.0 ± 9.2 nmoles/mg protein.
† Mean ± S.E.

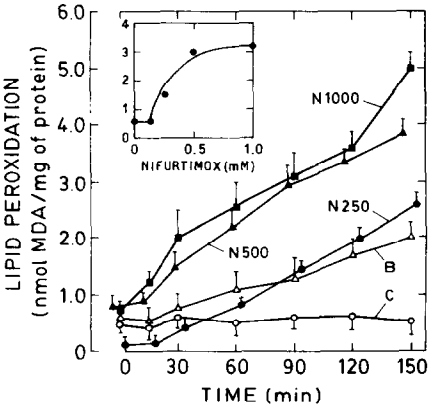

Fig. 3. Effects of nifurtimox (N) and benznidazole (B) on the generation of malondialdehyde in liver extracts supplemented with the NADPH-generating system. Conditions were as described in the legend of Fig. 1 and under Materials and Methods. P for 0-time samples, > 0.05. Inset: effect of increasing nifurtimox concentrations after 90 min of incubation.

duced a progressive diminution of GSH content which, after 150 min of incubation, was practically exhausted. Similar results were obtained with 250 and 500 μM nifurtimox, but, unexpectedly, in the 0–120 min incubation period somewhat lower effects were obtained with 1 mM nifurtimox. Nifurtimox at 10–100 μM did not affect GSH concentration (experimental data omitted). Benznidazole (2.0 mM) also decreased the GSH content, but it was less effective than nifurtimox, whatever the concentration of this latter in Fig. 1. It seems pertinent to note that in order to cause a significant decrease of GSH, nifurtimox and benznidazole were used at concentrations several-fold higher than those expected to be found in the blood of rats treated with pharmacological doses of the drug (10–20 μM nifurtimox; Ref. 17). Under these experimental conditions, the nitroheterocyclic drugs inhibited GSSG-reductase (Fig. 2) but, at the same concentrations, they did not inhibit GSH-peroxidase (experimental data omitted). In good agreement with these results, incubation of liver extract with the nitroheterocyclic drugs produced a decrease of GSH and an increase of GSSG, to about the same extent (Table 1).

Figure 3 shows the effects of nifurtimox and benznidazole on the production of TBA-reacting intermediates which, according to available data [18, 19], consisted essentially in malondialdehyde. The rate of malondialdehyde generation in the control sample remained constant throughout the incubation period, but addition of nifurtimox (or benznidazole) produced significant stimulation. Increase of the nifurtimox concentration from 250 to 500 μM almost doubled the rate of lipid peroxidation, but 1 mM nifurtimox did not increase it any further, thus showing saturation of the lipid peroxidating system (Fig. 3, inset). With 250 and 500 μM nifurtimox, the kinetics of the overall process showed an induction period, lasting for the first 20–30 min of incubation, but with the 1.0 mM concentration the lag was not observed. Similar experiments were performed using 10–100 μM nifurtimox, but at these concentrations the drug did not stimulate the production of TBA-reacting substances (Fig. 3, inset). The time lag was also observed with benznidazole, which, as expected from the variation of GSH content (Fig. 1 and Table 1), was less effective than nifurtimox. In good agreement with these results addition of GSH (excess) to the incubation mixture completely prevented malondialdehyde generation (Table 2).

To confirm the role of oxygen radicals, especially $OH^{\cdot}$ in the drug-induced lipid peroxidation, nifurtimox was assayed in the presence of radical scavengers. Benzoate(Na) and mannitol concentrations were selected, taking into account the observation of Que *et al.* [20] and Mak *et al.* [21]. The results in Table 3 show that superoxide dismutase produced complete inhibition of malondialdehyde generation. Lesser, though marked, effects were produced by catalase, benzoate, mannitol and L-histidine, in decreasing order of effectiveness. Possible non-specific protein effects by superoxide dismutase and catalase were ruled out, taking into account the relatively high content of liver protein in the incubation mixture (20 mg/ml).

Table 2. Effect of exogenous GSH on malondialdehyde generation by liver extracts supplemented with nifurtimox and benznidazole*

Drug (mM)	GSH (mM)	ΔA_{532}
Nifurtimox (1.0)	0	0.990 ± 0.040†
	5.0	0
Benznidazole (2.0)	0	0.226 ± 0.033
	5.0	0

* Experimental conditions were as in Fig. 3. Incubation time: 1 hr.
† Mean ± S.E.

Table 3. Effect of free-radical scavengers on nifurtimox-induced lipid peroxidation in liver extracts*

Addition	ΔA_{532}	Inhibition (%)
None	0.270 ± 0.018†	
Catalase (200 μg/ml)	0.010 ± 0.006	93.3
Superoxide dismutase (50 μg/ml)	0.0 ± 0.0	100.0
Mannitol (150 mM)	0.083 ± 0.01	70.0
Benzoate (150 mM)	0.057 ± 0.007	79.3
L-Histidine (10 mM)	0.120 ± 0.0	56.5

* Standard reaction mixture was supplemented with 500 μM nifurtimox and the addition indicated above. Incubation was for 2 hr; other experimental conditions were as described under Materials and Methods.

† Mean ± S.E.

DISCUSSION

Utilization of liver cell-free extracts for comparative measurements of GSH content and malondialdehyde generation enabled us to confirm and extend previous observations on the function of nifurtimox and benznidazole as "active oxygen" generators in rat liver, both *in vitro*, using microsomes [1–3], and *in vivo* [4]. The 9000 *g* supernatant fraction contained endoplasmic reticulum, mitochondrial membranes and cytosol and included, among other components, the microsomal NADPH–cytochrome P-450 (*c*) reductase (nitroreductase; NR), GSSG-reductase, GSH-peroxidase, superoxide dismutase (SOD), GSH and lipids. The decrease of GSH content in Fig. 1 and Table 1 may then be explained as follows: (a) Reaction 3, catalyzed by the microsomal nitroreductase, generated the nitro

$$\text{NADPH} + \text{ArNO}_2 \xrightarrow{\text{NR}} \text{NADP}^+ + \text{ArNO}_2^{\cdot-} + \text{H}^+ \quad (3)$$

$$\text{ArNO}^{\cdot-} + \text{O}_2 \longrightarrow \text{ArNO}_2 + \text{O}^{\cdot-} \quad (4)$$

$$\text{O}^{\cdot-} + \text{O}^{\cdot-}_2 + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2 \quad (5)$$

$$\text{GSH} + \text{O}_2\text{H} \longrightarrow \text{GS}^{\cdot} + \text{H}_2\text{O} \quad (6)$$

$$\text{GS}^{\cdot} + \text{GS}^{\cdot} \longrightarrow \text{GSSG} \quad (7)$$

anion radical [1, 3]; (b) Reaction 4 generated $O_2^{\cdot-}$ [1, 3] and prevented the reduction of the nitro radical to more reduced intermediates, capable of conjugating with GSH [22]; (c) Reaction 5 generated H_2O_2 [1, 3], which, according to Reaction 1 (GSH-peroxidase reaction), oxidized GSH to GSSG (Table 2); (d) the perhydroxyl radical ($O_2^{\cdot-}$ conjugate) oxidized GSH to GS$^{\cdot}$ (thiyl radical; Reaction 6) and GS$^{\cdot}$ dimerized to GSSG (Reaction 7) [23]; (e) nifurtimox and benznidazole inhibited Reaction 2 (the GSSG-reductase reaction; Fig. 2 and Ref. 4), an inhibition which, associated with the normal operation of the GSH-peroxidase reaction, contributed significantly to GSH depletion. Concerning the relative importance of Reactions 6 and 7 for GSH depletion, the possible competence of Reaction 5 (the superoxide dismutase reaction) with Reaction 6 is worth noting. The dismutase has a very high molecular activity ($k_4 = 2 \times 10^9\ \text{M}^{-1} \cdot \text{s}^{-1}$; Ref. 5) and, accordingly, direct oxidation of GSH (Reactions 6 + 7) might be of limited importance.

Catalase, superoxide dismutase, mannitol, benzoate and L-histidine inhibited malondialdehyde generation (Table 3), in good agreement with results by Zimmermann *et al.* [24], and Kellog and Fridovich [25, 26]. The scavenger effect supports the role of hydroxyl radicals (generated by Reaction 8) for lipid peroxidation.

$$\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{\cdot} + \text{OH}^- + \text{O}_2 \quad (8)$$

The O_2H radical might also initiate the peroxidative process (Reaction 9; RH, polyunsaturated fatty acid) [27] but, in the given experimental conditions, the role of this reaction was limited, as indicated by the strong effect of catalase in Table 3.

$$\text{RH} + \text{O}_2\text{H} \rightarrow \text{R}^{\cdot} + \text{H}_2\text{O}_2 \quad (9)$$

Taken together, the results presented in Figs. 1 and 2 may explain satisfactorily the absence of lipid peroxidation in liver of rats treated with pharmacological doses of nifurtimox [4]. Thus, physiological concentration of GSH should be quite sufficient to cope with the peroxidative challenge induced by nitroheterocyclic drugs, at intracellular concentrations in all probability much lower than in Figs. 1 and 3. Furthermore, GSH regeneration by the GSH-reductase (Reaction 2) would ensure a permanent supply of GSH for H_2O_2 and hydroperoxide detoxication. GSH depletion by itself might not, however, be a necessary requirement for the stimulation of the lipid peroxidative process, as shown by the effect of diethylmaleate, a powerful GSH-depleting agent [28]. Oxygen radical generation by redox-cycling of the nitroheterocyclic drugs should be the determinant factor for the peroxidative process, the extent of which would be enhanced by the reduction in the cellular antioxidant capacity, or vice versa, nullified by excess of the latter (Table 2). In this context, the effect of 1.0 mM nifurtimox in Fig. 3 is easily explained, since at that concentration the failure of the GSH-dependent protection allowed oxygen radicals to initiate lipid peroxidation without lag. A similar relationship between GSH content and lipid peroxidation has been observed in bromobenzene-treated hepatocytes [29], and also in relation to liver damage induced by ethanol [30, 31].

Acknowledgements—This work was supported by grants from the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, Organization of American States (Multinational Programme of Biochemistry), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Programa Nacional de Enfermedades Endémicas (SUBCYT) and Fundación Lucio Cherny, República Argentina. M. D. and A. O. M. S. are Career Investigators and S. G. G. is a Research Fellow of CONICET. The skilful technical assistance of Mr. E. Klimosvsky and Ms. Lilia T. Viñas is gratefully acknowledged.

REFERENCES

1. R. Docampo, S. N. J. Moreno and A. O. M. Stoppani, *Archs Biochem. Biophys.* **207**, 316 (1981).
2. S. N. J. Moreno, D. J. Palmero, K. Eiguchi de Palmero, R. Docampo and A. O. M. Stoppani, *Medicina (Bs. Aires)* **40**, 553 (1980).
3. S. N. J. Moreno, R. Docampo, R. P. Mason, W. Leon and A. O. M. Stoppani, *Archs Biochem. Biophys.* **218**, 585 (1982).
4. M. Dubin, S. N. J. Moreno, E. E. Martino, R. Docampo and A. O. M. Stoppani, *Biochem. Pharmac.* **32**, 483 (1983).
5. B. Chance, H. Sies and A. Boveris, *Physiol. Rev.* **59**, 527 (1979).
6. M. Nakano, Y. Tsutsumi and Y. Ushijima, *Biochim. biophys. Acta* **252**, 335 (1971).
7. G. L. Elıman, *Archs Biochem. Biophys.* **82**, 70 (1959).
8. J. Sedlack and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
9. M. S. Moron, J. W. Depierre and B. Mannervik, *Biochim. biophys. Acta* **582**, 67 (1979).
10. C. W. Owens and R. V. Belcher, *Biochem. J.* **94**, 705 (1965).
11. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
12. A. G. Gornall, G. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
13. R. E. Pinto and W. Bartley, *Biochem. J.* **115**, 449 (1969).
14. R. A. Lawrence and R. F. Burk, *Biochem. biophys. Res. Commun.* **71**, 952 (1976).
15. H. Sies, A. Wahllander, C. Waydhas, S. Soboll and D. Häberle, *Adv. Enzyme Regulat.* **18**, 303 (1980).
16. O. W. Griffith and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5606 (1979).
17. H. Medenwald, K. Brandan and K. Schlossmann. *Arzneimittel-Forsch.* **22**, 1613 (1972).
18. M. U. Dianzani and G. Ugazio, in *Biochemical Mechanisms of Liver Injury* (Ed. T. F. Slater), p. 669. Academic Press, London (1978).
19. R. A. Jordan and J. B. Schenkman, *Biochem. Pharmac.* **31**, 1393 (1982).
20. B. G. Que, K. M. Downey and A. G. So, *Biochemistry* **19**, 5987 (1980).
21. I. T. Mak, H. P. Misra and W. B. Weglicki, *J. biol. Chem.* **258**, 13733 (1983).
22. A. J. Varghese, *Biochem. biophys. Res. Commun.* **112**, 1013 (1983).
23. H. Wefers and H. Sies, *Eur. J. Biochem.* **137**, 1613 (1972).
24. R. Zimmermann, L. Flohé, U. Weser and H. J. Hartmann, *Fedn Eur. Biochem. Soc. Lett.* **29**, 117 (1973).
25. C. W. Kellog and I. Fridovich, *J. biol. Chem.* **250**, 8812 (1975).
26. C. W. Kellog and I. Fridovich, *J. biol. Chem.* **252**, 6721 (1977).
27. B. H. J. Bielski, R. L. Arudi and M. W. Sutherland, *J. biol. Chem.* **258**, 4759 (1983).
28. R. Reiter and A. Wendel, *Chem. Biol. Interact.* **40**, 365 (1982).
29. M. T. Smith, H. Thor and S. Orrenius, *Biochem. Pharmac.* **32**, 763 (1983).
30. M. Younes and C-P. Siegers, *Chem. Biol. Interact.* **34**, 257 (1981).
31. L. A. Videla and A. Valenzuela, *Life Sci.* **31**, 2395 (1982).