Biochemical Pharmacology, Vol. 32, No. 3, pp. 483–487, 1983.
Printed in Great Britain.

0006-2952/83/030483-05 $03.00/0

INCREASED BILIARY SECRETION AND LOSS OF HEPATIC GLUTATHIONE IN RAT LIVER AFTER NIFURTIMOX TREATMENT

MARTA DUBIN, SILVIA N. J. MORENO, ELBA E. MARTINO, ROBERTO DOCAMPO and A. O. M. STOPPANI*

Instituto de Química Biológica, Centro de Investigaciones Bioenergéticas, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

(*Received* 23 *April* 1982; *accepted* 7 *July* 1982)

Abstract—Treatment of rats with nifurtimox, a nitrofuran derivative widely used for the treatment of Chagas' disease, induced a time- and dose-dependent depletion of liver glutathione, maximal effects being obtained with 200 mg nifurtimox/kg body weight. Extra release of both oxidized (GSSG) and reduced (GSH) glutathione into bile contributed to this depletion. Glutathione excretion into bile accounted for only part of liver glutathione loss, thus indicating that, in addition to the GSH-peroxidase reaction (resulting in GSSG generation), other glutathione-related processes were involved in nifurtimox detoxification. Bile flow, bile salt excretion, liver lipid conjugated diene content, liver glutathione reductase and glutathione peroxidase activities, and serum alanine aminotransferase (ALAT) activity were not affected by the nifurtimox treatment, thus ruling out widespread damage of the liver cell by nifurtimox. Nevertheless, the extra GSH release in the nifurtimox-treated rats may indicate an alteration of the hepatocyte membrane.

Nifurtimox†, a nitrofuran derivative, is one of the most effective drugs used in the treatment of acute Chagas' disease. Nifurtimox chemotherapy, however, is limited by the occurrence of toxicity associated with treatment at total dosage levels [1–3].

According to Docampo *et al.* [4], addition of nifurtimox to liver microsomes in the presence of NADPH increases the generation of superoxide anion, hydrogen peroxide and lipoperoxides. It is a well-known fact that oxygen radicals and the products of lipoperoxidation, including malondialdehyde, cause damage to protoplasmic constituents at the molecular level and, among the molecular targets responsive to peroxidative attack, proteins represent the most susceptible one [5]. However, living cells have developed defensive mechanisms against such oxidative attacks. In the liver, the main physiological mechanism for hydrogen peroxide and lipoperoxide detoxification involves the glutathione redox cycle and, particularly, the GSH-peroxidase reaction [6]. This peroxidase catalyzes the conversion of lipid hydroperoxides (ROOH) to innocuous hydroxyacids (ROH) (Reaction 1)

$$ROOH + 2GSH \xrightarrow{\text{GSH-peroxidase}} ROH + H_2O + GSSG \quad (1)$$

and, concerning hydrogen peroxide, compartmentation in the cytosol and the mitochondria makes GSH-peroxidase (Reaction 2)

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-peroxidase}} 2H_2O_2 + GSSG \quad (2)$$

largely responsible for H_2O_2 decomposition in the liver cell [6]. As a result of the GSH-peroxidase reaction, GSH is converted to GSSG which is excreted into the bile [6, 7]. Hence, by measuring glutathione content in the liver and glutathione excretion into the bile it should be possible to confirm, *in vivo*, the above-mentioned effect of nifurtimox on hydrogen peroxide and hydroperoxide generation by liver microsomal preparations. This reasoning prompted the present study. To rule out possible general cytotoxic effects of nifurtimox, other variables of liver function such as bile flow, bile salt excretion [8], lipid conjugated diene content [5], and GSSG-reductase, GSH-peroxidase and alanine aminotransferase activities were measured in both nifurtimox-treated and control rats.

MATERIALS AND METHODS

Treatment of rats. Wistar male rats (200–250 g) from the National Institute of Pharmacology were used in the experiments. Animals were fed "Nutrimento" (Purina-like) rat chow and water *ad lib*. and were not fasted prior to use. The protein content of the diet was 23.4% and included all the essential amino acids. Room temperature was kept at 24° with a 12 hr cycle of light and dark. To minimize the influence of circadian variations, experiments were invariably initiated at 10:00 a.m. In all cases, nifurtimox was injected intraperitoneally, dissolved in

* To whom all correspondence should be addressed.

† Abbreviations: nifurtimox, 3-methyl-4-(5′-nitrofurfuryldene - amino) - tetrahydro - 4*H* - 1,4 - thiazone - 1,1′-dioxide; 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); ALAT, alanine aminotransferase (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase).

Tween 80–0.15 M NaCl (1:24, v/v). Unless stated otherwise, the nifurtimox dose was 200 mg/kg. Control rats received the corresponding volume of Tween 80–NaCl solution. For the study of the glutathione content of liver, the rats were injected with nifurtimox and decapitated, without anesthesia, at the times indicated in Fig. 1. The liver was then rapidly removed, weighed, and homogenized for glutathione measurement.

For the studies of nifurtimox effect on glutathione excretion into bile, rats were anesthetized with sodium pentobarbital (50 mg/kg body wt, intraperitoneally) and, after opening of the peritoneal cavity, the bile duct was cannulated with a PE10-catheter (Biotrol, Pharma, Paris) just before the hepatic hilus, in order to avoid contamination with pancreatic juice. The rectal temperature, controlled throughout the experimental period, was maintained between 37.5 and 38.5° using a heating table. The jugular vein, cannulated with polyethylene catheters, was used for infusions. A 0.15 M NaCl infusion was administered throughout the experiment at the rate of 7.5 μl/min, to compensate for the loss of water and electrolytes. Bile was collected at three 15-min intervals immediately after cannulation (basal period) and weighed to the nearest 0.10 mg. The values taken for each rat are the means of these measurements. No correction was made for specific gravity. Care was taken to transfer samples directly to 0°, and glutathione assays were run immediately. After the basal period, nifurtimox was administered as a single injection (final volume, 0.7 ml), the peritoneal cavity being closed after nifurtimox administration. Thenceforth, bile flow was measured every 15 min, and bile sampling was carried out as described above. Bile salts were measured in the same samples. At the end of the experiment the liver was rapidly removed and weighed. No mortality was observed in the nifurtimox-treated or control rats during these experiments.

Glutathione assays. The glutathione content in liver cytosol was measured. The liver was suspended in 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 9,000 *g* for 10 min at 5°, and the supernatant fraction was further centrifuged at 105,000 *g* for 60 min at 2–3°. GSH was determined in the supernatant fraction by the method of Ellman [9], as modified by Sedlack and Lindsay [10]. No difference was detected when the resulting values were compared with those obtained by the enzymatic method [11].

GSSG in bile was measured by following NADPH oxidation in the presence of GSSG-reductase. The reaction mixture contained 50 mM phosphate buffer (K_2HPO_4–KH_2PO_4, pH 7.0), 0.5 mM NADPH, 0.1 unit/ml of yeast GSSG-reductase and a 50–100 μl bile sample, in a final volume of 3 ml. Total glutathione, i.e. GSH plus 2GSSG, was determined in bile samples using GSSG-reductase and 5,5-dithiobis-(nitrobenzoic acid), as described in Refs. 12 and 13. The reaction mixture contained 50 mM phosphate buffer (as above), 0.1 unit/ml GSSG-reductase, 0.5 mM NADPH, 33 μM 5,5′-dithiobis-(nitrobenzoic acid) and 1 μl bile, 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. A Perkin–Elmer 550-S spectrophotometer was used for all these measurements.

Determination of enzymes. The activities of GSSG-reductase and GSH-peroxidase were measured at 30°. Liver homogenates were made in 50 mM phosphate, 1 mM EDTA (pH 7.0), 1% Triton X-100 and 50 mM phosphate buffer (as above) in the proportion of 10 ml/g liver; after centrifugation at 105,000 *g* for 60 min, the supernatant fraction was saved for the enzyme assay. GSSG-reductase was measured essentially as described in Ref. 14. The reaction mixture contained 50 mM phosphate buffer (as above), 0.5 to 1.0 mM GSSG, 0.5 mM NADPH and 5–10 μl of liver supernatant fraction; final volume was 3 ml. The same reaction mixture was used to assay nifurtimox inhibition of GSSG-reductase. GSH-peroxidase was measured by a modification of the coupled assay procedure [14, 15]. 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 2–3 μl liver supernatant fraction; final volume was 3 ml. In all cases NADPH oxidation was followed by measuring the decrease of absorption at 340 nm. Serum ALAT was measured by the Reitman–Frankel method [16] on 100 μl blood samples obtained from the inferior vena cava at the end of the experiment. Measurements were made using a Perkin–Elmer 550-S spectrophotometer.

Analytical methods. Biliary bile salt concentration was measured by the enzymatic method using a 3α-hydroxysteroid dehydrogenase [17]. Protein was determined by the biuret method [18].

Lipid peroxidation. Lipid peroxidation *in vivo* was estimated by measuring the conjugated diene content by ultraviolet absorption of lipid extracts of the liver homogenates, as described by Klaassen and Plaa [19]. The total volume of the lipid extract was 10 ml, corresponding to a 20 mg/ml sample of liver homogenate.

Reagents. These were obtained from the following sources: nifurtimox from Bayer A.G., Leverkusen, German Federal Republic; Tween 80, 5,5′-dithiobis-(nitrobenzoic acid), NADPH, GSSG-glutathione reductase, 3α-hydroxysteroid dehydrogenase, and *tert*-butyl hydroperoxide from the Sigma Chemical Co., St. Louis, MO. Other reagents were of analytical grade.

Statistical analysis. This was performed using Student's *t*-test.

RESULTS

Figure 1 shows liver glutathione content for nifurtimox-treated and control rats. Initial values agree well with those reported by other workers [20, 21] and essentially represent GSH. In the nifurtimox-treated rats, the glutathione content decreased significantly, maximal diminution being observed 2–4 hr after nifurtimox injection. Nifurti-

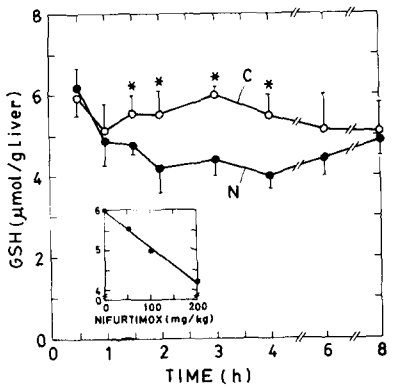

Fig. 1. Liver glutathione content in control (C) and nifurtimox-treated (N) rats. Each value is the mean from six experiments; the bars over the points represent S.E. The experimental conditions are described in the text. An asterisk indicates $P < 0.02$ except for the 3-hr sample where P was <0.05. Other differences were not significant. Inset: effect of various doses of nifurtimox.

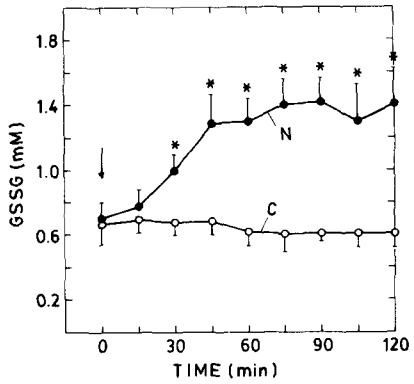

Fig. 2. Oxidized (GSSG) glutathione excretion into bile, in control (C) and nifurtimox-treated (N) rats. Conditions are described in the text and in the legend of Fig. 1. The arrow indicates the time of nifurtimox injection. An asterisk indicates $P < 0.001$. Other differences were not significant.

mox effect was dose-dependent and, as shown by the inset in Fig. 1, maximal glutathione depletion was observed with the 200 mg/kg dose. With this dose, the liver glutathione content decreased by 30%, a value equivalent to 80 μmoles glutathione for the whole liver.

Figures 2 and 3 show GSSG and GSH concentrations in the bile of control and nifurtimox-treated rats; initial values confirm those previously reported [22]. As expected from the results in Fig. 1, biliary GSSG increased significantly after nifurtimox administration (Fig. 2), maximal increase occurring in about 90 min. Figure 3 shows a similar variation in GSH concentration. Comparison of GSSG and GSH values in Figs. 2 and 3 shows a 2-fold increase of both forms, as a result of nifurtimox administration. Since bile flow was not modified significantly during the 2-hr period after nifurtimox administration (Table 1), these results prove that nifurtimox increased the efflux of both GSSG and GSH from the hepatocyte. The GSSG/(GSH + 2GSSG) ratio was approximately 0.26, without significant difference between nifurtimox-treated and control rats.

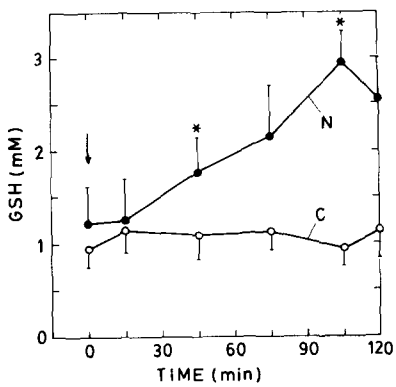

Fig. 3. Reduced (GSH) glutathione excretion into bile, in control (C) and nifurtimox-treated (N) rats. Conditions are described in the text and in the legend of Fig. 1. The arrow indicates the time of nifurtimox injection. An asterisk indicates $P < 0.05$ (45-min sample) or 0.01 (105-min sample). Other differences were not significant.

Table 1. Bile flow, bile salt excretion, enzyme activities and conjugated diene content in control and nifurtimox-treated rats*

Variable	Values in rats	
	Control	Nifurtimox-treated
Liver GSH-peroxidase (units/mg protein)	1.12 ± 0.16†	1.03 ± 0.15†
Liver GSSG-reductase (units/mg protein)	0.169 ± 0.043	0.142 ± 0.034
Serum alanine aminotransferase (units/l)	32.3 ± 5.7	40.7 ± 3.5
Conjugated diene absorbance in lipid extract (ΔA_{235})	0.23 ± 0.01	0.23 ± 0.01
	Basal period	After nifurtimox‡ (2 hr)
Bile flow (μl/min/kg)	94 ± 25	88 ± 12
Bile salt excretion (μmoles/min/kg)	2.71 ± 0.40	2.60 ± 0.30

* Experimental conditions are described under Materials and Methods. Rats were treated with 200 mg nifurtimox/kg.

† Mean ± S.D. (four to six independent determinations).

‡ Values obtained at the various times after nifurtimox administration did not show significant differences with respect to the basal period or the 2-hr values.

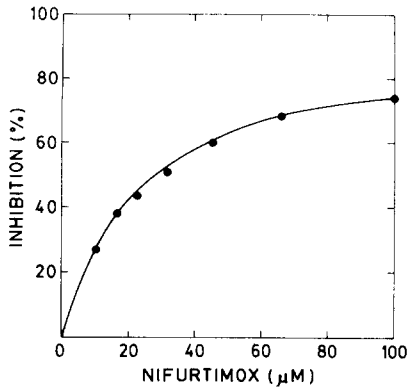

Fig. 4. Inhibition of liver glutathione reductase by nifurtimox, *in vitro*. Experimental conditions are described under Materials and Methods.

Two hours after nifurtimox administration, total GSSG excretion into bile (in μmoles) was 1.3 (control rats) and 3.0 (nifurtimox rats), while the corresponding values for GSH were 2.6 and 5.4 respectively [these are maximal values since calculations were based on GSSG (or GSH) concentration in the 2-hr samples]. Accordingly, total glutathione (GSH + 2GSSG) excretion was (in μmoles GSH) 5.2 (control rats) and 11.4 (nifurtimox rats).

Other measurements. To establish whether the nifurtimox treatment modified the activity of liver enzymes involved in glutathione metabolism or other variables of liver function, GSH-peroxidase, GSSG-reductase, ALAT, bile flow, bile salt excretion and conjugated diene content in liver lipids were measured in nifurtimox-treated and control rats. The results in Table 1 do not show significant differences between the two groups. Despite the normal value of the GSH-reductase activity in the nifurtimox-treated rats, nifurtimox inhibited the enzyme *in vitro*, as described in Fig. 4.

DISCUSSION

Total liver glutathione (about 6 μmoles/g liver; 71–95% GSH) involves a "labile" pool (half-life time, 1.7 hr; pool size 30% of total) and a more "stable" pool (half-life time 15 hr; pool size 70% of total) [23, 24]. Assuming that in our experiments this glutathione distribution was valid, recalculation of data in Fig. 1 demonstrates that 2 hr after nifurtimox administration the "labile" pool (1.7 μmoles glutathione/g liver) should have been completely depleted.

The decrease of liver glutathione after nifurtimox administration (Fig. 1) was in accord with the extra release of both GSSG (Fig. 2) and GSH (Fig. 3) into bile. Release of GSSG may be taken as a demonstration of nifurtimox-dependent H_2O_2 generation in the liver cells since studies with perfused liver have shown, first, that the rate of the GSH-peroxidase reaction in the hepatocyte is a function of the H_2O_2 intracellular concentration and, second, that, once formed, GSSG is acted on by the biliary excretory system and excreted into bile [6, 21]. Considering the specificity of the GSH-peroxidase reaction [6], it is possible that the extra release of GSSG would also depend on hydroperoxides formed by nifurtimox-induced lipoperoxidation reactions. The contribution of hydroperoxides to the extra GSSG release, however, was opposed by the normal conjugated diene content of the liver lipids from the nifurtimox-treated rats (Table 1). Finally, the extra release of GSSG might also have involved a decreased rate of the GSSG-reductase reaction (Reaction 2). This effect could have been due to enzyme inhibition by nifurtimox (Fig. 4) and/or nifurtimox-induced decrease of NADPH concentration in the hepatocyte [4]. This possibility is not supported by either the unmodified value of the GSSG/GSH + 2GSSG ratio, or the normal GSH-reductase activity in the nifurtimox-treated rats (Table 1). Validity of the latter point can, however, be questioned, because of the reversibility of nitrofuran inhibition of the GSSG-reductase [25].

Comparison of liver glutathione depletion values and total glutathione excretion into bile shows a large discrepancy, since 2 hr after nifurtimox administration GSH + 2GSSG excretion represented only 5% of the liver glutathione loss. The discrepancy indicates that, in addition to the GSH-peroxidase reaction, other nifurtimox-induced metabolic processes might have contributed to the GSH depletion. As suggested for the paraquat-induced depletion of liver glutathione [26], it is possible that in the nifurtimox-treated rats GSH acted as a scavenger [27] of nifurtimox-generated free radicals [28]. The resulting thiyl ($GS^{\cdot}$) radicals could then undergo uncontrolled reactions with protein SH-groups, which would lead to an apparent loss of hepatic GSH, since protein-bound glutathione escapes determination by the assay method.

Experiments with perfused liver have shown that release of GSH from the hepatocyte does not normally exceed the basal level on peroxide addition [6, 29]. This lack of response is regarded as strong evidence that the cell remains undamaged [6]. Accordingly, the extra GSH release in the nifurtimox-treated rats in Fig. 3 may indicate an alteration of the hepatocyte membrane. The exaggerated GSH release constrasts, however, with the normal values of the variables listed in Table 1, some of which (bile flow, biliary salt excretion, conjugated diene content and ALAT activity) are sensitive to lipoperoxide damage of the liver cell. Interestingly enough, treatment of rats with nifurtimox (200 mg per kg per day for 3 days) yielded results identical with those reported in Table 1, but a mild (30%) choleretic effect, due to the increase of the bile-salt-independent bile flow, was observed (unpublished observations). Although Chagas' patients under 1–2 months of treatment with nifurtimox on doses of 4–15 mg per kg per day, appeared to show no signs of liver pathology (in good agreement with our results in Table 1), the observations reported here point to the conclusion that, as with other mammalian tissues (brain, testes, etc.) [30], nifurtimox may also be toxic for the liver.

Acknowledgements—This work received financial support from the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical

Diseases, from PNUD/UNESCO (Grant 28), from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET), and Secretaría de Estado de Ciencia y Tecnología, Argentina. M. D., R. D. and A. O. M. S. are Career Investigators of CONICET, and S. N. J. M. is a Fellow of CONICET. We are grateful to Dr. H. Sies and T. P. M. Akerboom for useful discussions and to Dr. A. Haberkorn (Bayer A.G.) for supplying nifurtimox.

REFERENCES

1. H. S. Lugones, F. Peralto, D. Canal Feijóo and A. E. A. de Marteleur, *Boln chil. Parasit.* **24**, 19 (1969).
2. J. Cançado, A. A. Salgado, S. M. Batista and C. Chiari, *Revta goiana Med.* **22**, 203 (1976).
3. H. Van Den Bossche, *Nature, Lond.* **173**, 626 (1978).
4. R. Docampo, S. N. J. Moreno and A. O. M. Stoppani, *Archs Biochem. Biophys.* **207**, 316 (1981).
5. M. U. Dianzani and G. Ugazio, in *Biochemical Mechanisms of Liver Injury* (Ed. T. F. Slater), p. 669. Academic Press, London (1978).
6. B. Chance, H. Sies and A. Boveris, *Physiol. Rev.* **59**, 527 (1978).
7. H. Sies, A. Wahlländer and C. Waydhas, in *Functions of Glutathione in Liver and Kidney* (Eds. H. Sies and A. Wendel), p. 120. Springer, Berlin (1978).
8. G. L. Plaa and B. G. Priestly, *Pharmac. Rev.* **28**, 207 (1977).
9. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
10. J. Sedlack and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
11. M. S. Moron, J. W. Depierre and B. Mannervik, *Biochim. biophys. Acta* **582**, 67 (1979).
12. C. W. Owens and R. V. Belcher, *Biochem. J.* **94**, 705 (1965).
13. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
14. R. E. Pinto and W. Bartley, *Biochem. J.* **115**, 449 (1969).
15. R. A. Lawrence and R. F. Burk, *Biochem. biophys. Res. Commun.* **71**, 952 (1976).
16. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 171 (1957).
17. P. Talalay, *Meth. biochem. Analysis* **8**, 119 (1960).
18. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
19. C. D. Klaassen and G. L. Plaa, *Biochem. Pharmac.* **18**, 2019 (1969).
20. O. W. Griffith and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5606 (1979).
21. H. Sies, A. Wahlländer, C. Waydhas, S. Soboll and D. Häberle, *Adv. Enzyme Regulat.* **18**, 303 (1980).
22. H. Sies, O. Koch, E. Martino and A. Boveris, *Fedn Eur. Biochem. Soc. Lett.* **103**, 287 (1979).
23. N. Tateishi and T. Higashi, in *Functions of Glutathione in Liver and Kidney* (Ed. H. Sies and A. Wendel), p. 3. Springer, Berlin (1978).
24. G. Harisch, J. Eikemeyer and J. Schole, *Experientia* **35**, 719 (1979).
25. J. A. Buzzard and F. Kopko, *J. biol. Chem.* **238**, 464 (1963).
26. R. Brigelius and M. S. Anwer, *Res. Commun. Chem. Path. Pharmac.* **31**, 493 (1981).
27. T. Ohnishi, H. Yamazaki, T. Iyanagi, T. Nakamura and I. Yamazaki, *Biochim. biophys. Acta* **172**, 357 (1969).
28. R. Docampo, R. P. Mason, G. Mottley and R. P. A. Muniz, *J. biol. Chem.* **256**, 10930 (1981).
29. G. M. Bartoli and H. Sies, *Fedn Eur. Biochem. Soc.* **86**, 89 (1978).
30. S. N. J. Moreno, D. J. Palmero, K. Eiguchi de Palmero, R. Docampo and A. O. M. Stoppani, *Medicina, B. Aires* **40**, 553 (1980).