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The effect of a ferric iron complex on isolated rat-liver mitochondria. III. Mechanistic aspects of iron-induced calcium efflux

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Addition of iron(III)-gluconate complex to isolated rat liver mitochondria induced a net efflux of Ca^{2+} which was not inhibited by ruthenium red. This process resulted in the enhancement of Ca^{2+} cycling and a consequent membrane potential drop. Under these experimental conditions the content of mitochondrial glutathione did not appear to be critically modified, whereas an extensive oxidation of mitochondrial pyridine nucleotides was parallelly detected. Iron failed to induce appreciable changes in the oxidation level of pyridine nucleotides in mitochondria isolated from rats fed a selenium deficient diet, a condition in which mitochondrial glutathione peroxidase resulted inhibited by 80%. The iron-induced Ca^{2+} release in Se-deficient mitochondria appeared largely delayed and the membrane potential of these mitochondria did not present gross alterations. Iron was also found to induce a transient increase in the mitochondrial cyanide-insensitive oxygen consumption. This effect was largely prevented by the addition of the hydrogen peroxide scavenger catalase. It was concluded that iron induced the activation of a specific Ca^{2+} efflux pathway via the oxidation of pyridine nucleotides due to the hydrogen peroxide metabolism by glutathione enzyme system.

Introduction

The mechanism of hepatocellular damage in iron overload has not been clearly established [1,2]. Iron might act by inducing peroxidative reactions in cell membrane lipids [3,4] and/or by disrupting lysosomal membranes with release of cell-damaging hydrolytic enzymes [5].

Recent findings in this vein may suggest a possible primary involvement of liver mitochondria in causing cell damage in hepatic experimental iron overload [6–8]. Indeed some defects in the function of mitochondria isolated from siderotic rats associated with the in vivo occurrence of lipoperoxidative reactions in the mitochondrial membranes have been recently revealed [6,8,9]. In order to give a better insight into the mechanism of in vivo iron hepatotoxicity, studies from our laboratory have been performed on the in vitro effect of iron(III)-gluconate, a ferric complex used to induce experimental iron overload [6,7], on the functional integrity of isolated rat liver mitochon-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; buffer 1, 210 mM mannitol/70 mM sucrose/10 mM Hepes (pH 7.4); TPP, tetraphenylphosphonium; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GSH, reduced glutathione; $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside; H_2O_2, hydrogen peroxide.

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dria. Iron, which in this form was accumulated in mitochondria by an energy-independent process, broght about the induction of lipid peroxidation in mitochondrial membranes. This process was found to be associated with the activation of a rather specific Ca^{2+} efflux from mitochondria. Indeed, Ca^{2+} release did not result from irreversible damages to the inner membrane but as a consequence of rather subtle modifications of it. A continuous and energy draining cycling of Ca^{2+} resulting from this process led to the mitochondrial transmembrane potential drop [10–13]. As to this problem, it has also recently been found that the induction by $FeSO_4$ of lipid peroxidation in liver mitochondria was associated with a decrease in membrane potential and a derangement of Ca^{2+} transport [14].

In the present research we have investigated the biochemical mechanism underlying the in vitro activation of Ca^{2+} release from liver mitochondria by iron(III)-gluconate complex.

Materials and Methods

Iron(III)-gluconate complex (Ferlixit) was purchased from Natterman, Köln (F.R.G.). Bovine liver catalase was obtained from Boehringer Mannheim (Milano, Italy).

Animals were killed by decapitation after an overnigh starvation period. Liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure [15]. The selenium deficient mitochondria were isolated from rats fed on a selenium-deficient diet for 8 weeks [16]. The protein content of the final mitochondrial suspension was determined by the biuret method with bovine serum albumin as the standard.

The oxygen consumption was measured with a polarographic Clark-type oxygen electrode at 25 °C in a final volume of 3 ml. The concentration of mitochondria was 3 mg protein per ml. The incubation standard medium was as follows: 210 mM mannitol/70 mM sucrose/10 mM Hepes (pH 7.4) (buffer 1), containing 2 μM rotenone and 2 mM succinate as respiratory substrate.

The transmembrane potential ($\Delta\psi$) was measured at 25 °C in a final volume of 1.5 ml by monitoring with a tetraphenylphosphonium (TPP^+)-selective electrode, the movements of tetraphenylphosphonium across the mitochondrial membrane as in Ref. 17. An inner mitochondrial volume of 1.1 μl/mg protein was assumed. Mitochondria, 3 mg protein/ml, were incubated at 25 °C in buffer 1 containing 2 μM rotenone in the presence of 20 μM TPP^+ and then energized with 2 mM succinate.

Ca^{2+} movements across the inner mitochondrial membrane were followed by a Ca^{2+}-selective electrode as described in detail in Refs. 18 and 19. Mitochondria, 3 mg protein/ml, were incubated at 25 °C in buffer 1 containing 5 μM rotenone and 100 μM $CaCl_2$ in a final volume of 1.8 ml. After a preincubation period of 5 min, the initial rates of Ca^{2+} uptake were measured following the addition of 5 mM K^+-succinate as respiratory substrate.

Mitochondrial GSH content was measured as follows: mitochondria, 6.5 mg protein/ml, were incubated at 25 °C in buffer 1 containing 2 μM rotenone with succiante as the substrate. At timed intervals samples of 2 ml were taken and the reaction was stopped by rapid mixing with 1 ml of 5% trichloroacetic acid (wt/v) and 5 mM EDTA. After centrifugation aliquots of supernatant were reacted with Ellman's reagent as described in Ref. 20.

The redox state of mitochondrial pyridine nucleotides was measured fluorometrically as in Ref. 21 (340 nm excitation and 465 nm emission) in an Optica Fluorescence Spectrophotometer Model 115, Mitochondria, 2 mg/ml, were incubated at 25 °C in buffer 1 containing 2 μM rotenone in the presence of 100 μM $CaCl_2$ and succinate as respiratory substrate. In some experiments NADH and NAD(P)H were determined enzymatically using lactate dehydrogenase and glutathione reductase as described by Beatrice et al. [22]. In this case the fluorescence was compared to that obtained adding known amounts of NADH and NAD(P)H as internal standards.

Results

Fig. 1 shows that addition of iron(III)-gluconate complex, at a concentration of 0.6 mM, induces, after a brief lag period, a net release of Ca^{2+} from liver mitochondria which had previously accumulated a low pulse of Ca^{2+}, i.e., 33 nmol/mg protein. The onset and the rate of Ca^{2+}

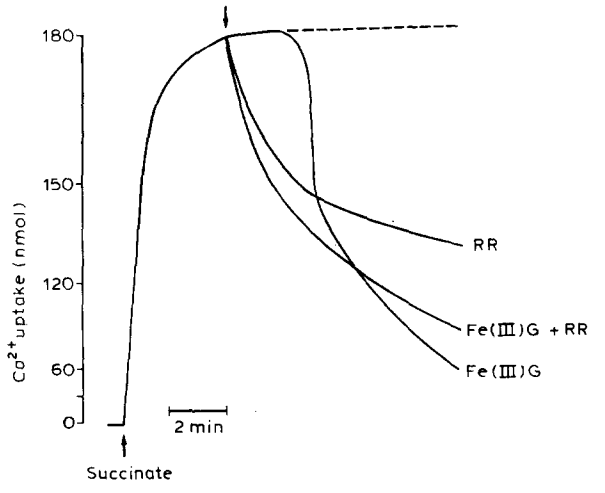

Fig. 1. Iron-induced Ca^{2+} efflux from rat-liver mitochondria. Mitochondria were loaded with 33 nmol Ca^{2+}/mg protein in the presence of succinate (succ). At the arrow either 2 μM ruthenium red (RR), 0.6 mM iron(III)-gluconate Fe(III)G, or ruthenium red together with iron (Fe(III)G + RR) were added. Results of a typical experiment are presented.

release appeared to be dependent on iron concentration up to an iron concentration of 0.1 mM (not shown). It also appears from the figure that the route by which Ca^{2+} is released is not inhibited by ruthenium red, a specific inhibitor of the electrogenic calcium uptake uniport [23]. Indeed ruthenium red, when added alone causes a slight release of Ca^{2+} which is typical of liver mitochondria [18]. On the contrary, when ruthenium red is added together with iron there is an immediate and more pronounced Ca^{2+} efflux.

A progressive drop of the membrane potential appears to be the functional implication of this event. Indeed addition of iron to mitochondria pre-loaded with the same low pulse of Ca^{2+}, induces a decrease of $\Delta\psi$ (Fig. 2A). Upon addition of EGTA, a specific Ca^{2+} chelator, the membrane potential immediatley returns to normal values. In order to test directly whether the restitutive effect of EGTA is due to the prevention of Ca^{2+} cycling, complexation of iron or both, experiments

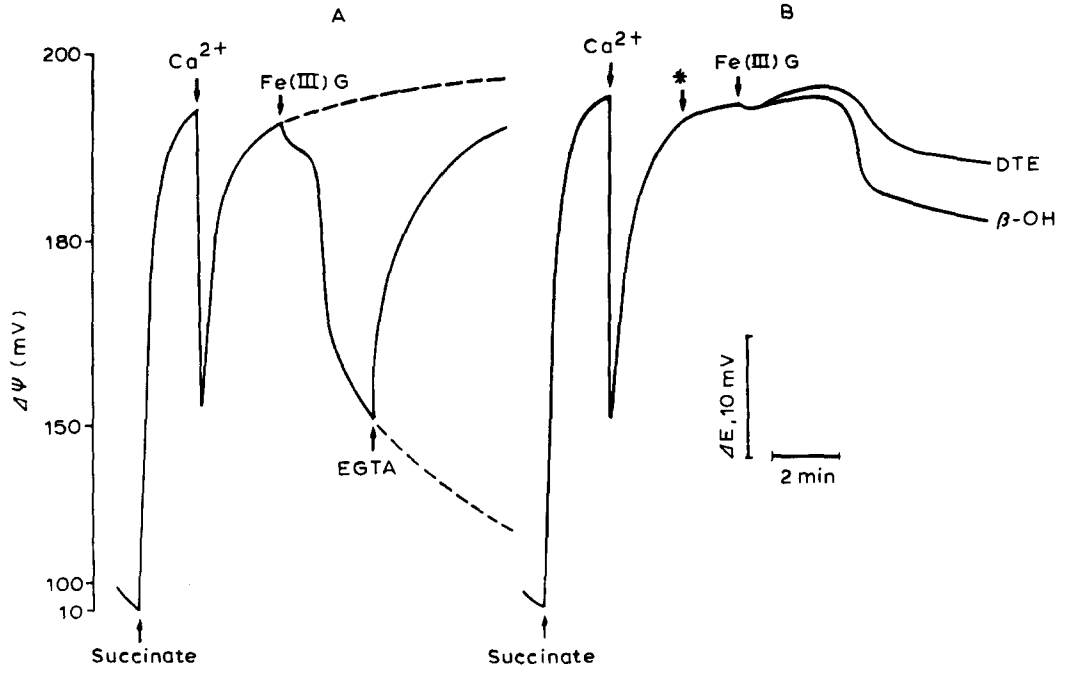

Fig. 2. Effect of iron on the mitochondrial transmembrane potential. Mitochondria were energized by the addition of 2 mM succinate. The arrows indicate the following additions: 33 nmol Ca^{2+}/mg protein (Ca^{2+}); 0.6 mM iron(III)-gluconate (Fe(III)G); 0.5 mM EGTA; 2 mM β-hydroxybutyrate (β-OH) and 2 mM dithioerythritol (DTE). The dashed lines in Fig. 2A indicate the membrane potential trace when no iron (upper trace) and no EGTA (lower trace) were added. ΔE, electrode potential. All other conditions as in Fig. 1.

were performed by using dexferrioxamine, a specific chelator of iron, instead of EGTA. The finding that this agent was without effect on the membrane potential trace, in agreement wih previous results [11], clearly indicates that Ca^{2+} cycling is the energy-dissipating process responsible for $\Delta\psi$ drop. It also appears from this figure (Fig. 2B) that supplying mitochondria with either β-hydroxybutyrate or dithioerythritol, which maintain the mitochondrial pyridine nucleotides and sulphydryl groups in a more reduced state, largely prevents the drop of $\Delta\psi$ caused by iron.

On the ground of these findings it may be derived a possible involvement of these two factors, i.e., the redox state of mitochondrial pyridine nucleotides and/or of thiol groups, in the mechanism of iron-induced release of Ca^{2+}. Iron was found to cause a decrease of the order of 20% in the mitochondrial content of reduced glutathione, taken as an index of the level of mitochondrial thiol groups, only after 8 min of incubation (not shown). At that time Ca^{2+} release had already been occurred.

On the contrary, iron induces an immediate and extensive oxidation of mitochondrial pyridine nucleotides (Fig. 3). This indicates that the oxidation level of pyridine nucleotides is responsible for iron-induced release of Ca^{2+}. It is to be noted that this ferric iron complex did not cause non enzymatic oxidation of pyridine nucleotides (not shown). From the same figure it also appears that iron fails to induce appreciable pyridine nucleotides oxidation in mitochondria isolated from selenium-deficient rats, a condition in which glutathione peroxidase activity was found to be inhibited by approx. 80% (not shown). Relevant to this problem, a reduction by far more than 60% in the content of reduced pyridine nucleotides was revealed by using enzymatic analysis in control mitochondria after 4 min incubation under the experimental conditions as in Fig. 4, whereas a decrease of the order of 15% appeared in Se-deficient mitochondria (not shown). These data, confirming the results reported in Fig. 3, exclude the possibility of misleading effect by quenching and scattering, possibly occurring in fluorescence measurements.

The results presented in Fig. 4 provide direct experimental evidence to the above proposal that

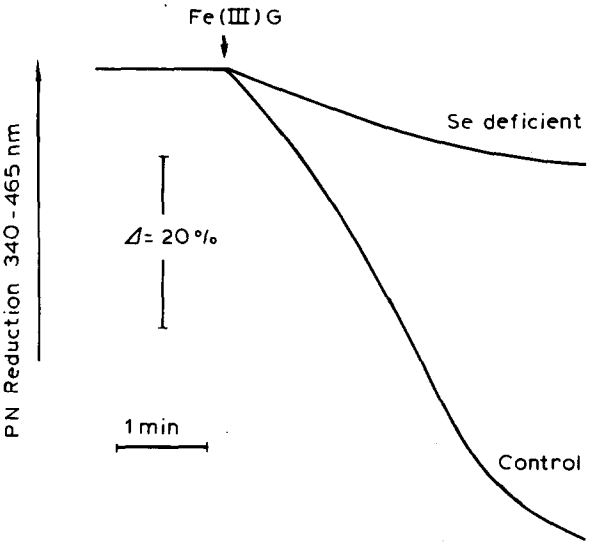

Fig. 3. Effect of iron on the redox state of mitochondrial pyridine nucleotides. Either control mitochondria (Control) or mitochondria isolated from selenium-deficient rats (Se deficient) were incubated at 25°C in 2 ml of buffer 1 containing 100 μM $CaCl_2$. At the arrow 0.6 mM iron(III)-gluconate (Fe(III)G) was added. PN, pyridine nucleotides. The absolute values, determined by enzymatic analysis, of the endogenous content of NADH plus NADPH, expressed as nmol per mg protein, were 3.2 ± 0.5 plus 4.7 ± 0.4 in control and 3.3 ± 0.4 plus 4.6 ± 0.6 in Se-deficient mitochondria ($n = 4$). These values for Se-deficient mitochondria were not significant in comparison with control, as assessed by Student's t-test.

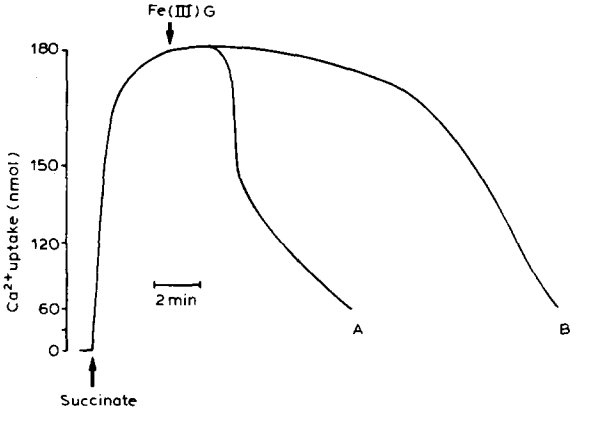

Fig. 4. Influence of selenium deficiency on Ca^{2+} release from rat liver mitochondria. Experimental conditions were exactly as in Fig. 1. (A) Control mitochondria; (B) Se-deficient mitochondria. All other conditions as in Fig. 1.

the iron-induced release of Ca^{2+} is due to a shift of pyridine nucleotides redox level. In fact, it can be seen that in Se-deficient mitochondria, the efflux of Ca^{2+} following iron addition is largely delayed. In agreement with this result it was also found that the presence of β-hydroxybutyrate and dithioerythritol, which prevented $\Delta\psi$ drop, retarted the release of Ca^{2+} from control mitochondria (not shown).

In agreement with this conclusion are also the results presented in Fig. 5. In fact, it appears that iron does not cause gross alterations in the membrane potential trace of Se-deficient mitochondria.

Fig. 6 shows that addition of iron(III)-gluconate complex to liver mitochondria, the respiratory chain of which was inhibited by 1 mM cyanide, induces a burst in the oxygen consumption (trace A). Reduction of ferric iron to ferrous iron and its subsequent reoxidation results in redox cycles which give rise to oxygen consumption other than that via cytochrome oxidase. No stimulation of O_2 consumption was observed in the absence of mitochondria (not shown). Thus excluding the possibility that impurities of ferrous iron in this ferric complex may account for this

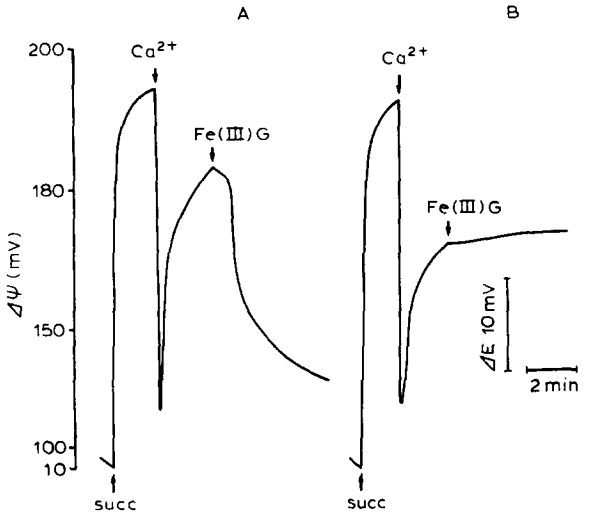

Fig. 5. Effect of iron on the membrane potential of control (A) and Se-deficient mitochondria (B). Mitochondria were energized by the addition of 2 mM succinate (succ). The arrows indicate the following additions: 33 nmol Ca^{2+}/mg protein (Ca^{2+}) and 0.6 mM iron(III)-gluconate (Fe(III)G). ΔE, electrode potential.

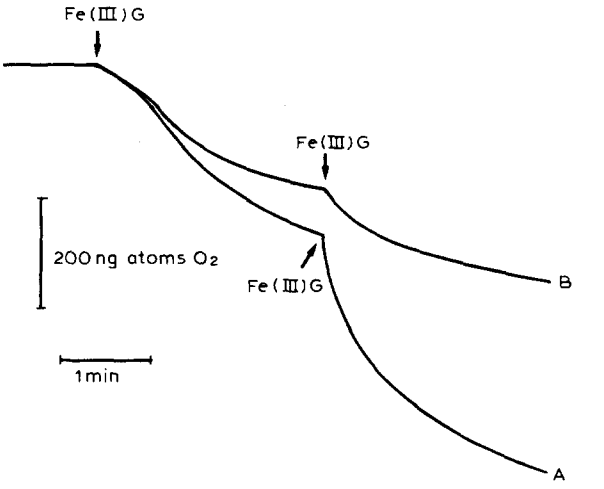

Fig. 6. Cyanide-insensitive oxygen consumption by rat-liver mitochondria induced by iron. Mitochondria were incubated at 25°C in buffer 1 containing 1 mM KCN. The arrows in trace A indicate the addition of 0.6 mM iron(III)-gluconate (Fe(III)G). The arrows in trace B indicate the addition of 0.6 mM iron(III)-gluconate together with 100 μg bovine liver catalase (Fe(III)G). The oxygen consumption was measured with a Clark-type oxygen electrode.

event. The same figure also shows that when the H_2O_2 scavenger, catalase, is added together with iron (trace B), it largely prevents this extra oxygen consumption.

Discussion

Addition of iron(III)-gluconate complex to isolated rat liver mitochondria induces the activation of Ca^{2+} release through a pathway which is not inhibited by ruthenium red (see Fig. 1), a specific inhibitor of electrogenic Ca^{2+} uptake (uniport). This indicates that Ca^{2+} is released via a distinct electroneutral antiport, according to Ref. 24. Should Ca^{2+} be released by the reversal of the electrogenic uptake pump, the Ca^{2+} efflux would be inhibited by ruthenium red. The iron-induced Ca^{2+} efflux results in enhancement of Ca^{2+} cycling, which is the energy-dissipating process responsible for the observed decrease of membrane potential (see Fig. 2). Indeed, when this process is blocked with EGTA, the inner membrane immediately repolarizes. This excludes the possibility of non-specific increase in inner membrane permeability by iron, i.e., Ca^{2+} release is the cause

for and not the consequence of $\Delta\psi$ drop.

The iron-induced release of Ca^{2+} appears to be linked to the oxidation of pyridine nucleotides, a condition which, as first suggested by Lehninger et al. [25], has been reported to induce the activation of a specific Ca^{2+} release route following the addition to liver mitochondria of different toxic agents [21,26–32]. On the contrary, the redox state of mitochondrial thiol groups, which has also been implicated in modulating the capability of mitochondria to retain Ca^{2+} [22,33–36], does not seem to be critically involved in this mechanism. This conclusion may be derived by evaluating the effect of iron on the mitochondrial content of reduced glutathione. Indeed, a glutathione depletion to an extent which may induce Ca^{2+} efflux [36] was found only when the process of Ca^{2+} release had been occurred completely.

As to the biochemical mechanism underlying the mode of action of this ferric complex the present data show that the oxidation of pyridine nucleotides as well as the consequent Ca^{2+} efflux involve to a large extent the mitochondrial glutathione peroxidase. In fact, when the activity of this enzyme is inhibited by a selenium-deficient diet, the iron-induced pyridine nucleotides oxidation and Ca^{2+} release are largely prevented. These results also indicate that the action of iron on the pyridine nucleotides redox state is indirect. Indeed the observation that the oxidation of pyridine nucleotides is sensitive to alterations of glutathione enzyme system suggests that the metabolism of hydroperoxidic products by iron via glutathione peroxidase, glutathione reductase and the energy-linked transhydrogenase [16] is involved in this process. Specifically hydrogen peroxide appears to be the reactive oxygen form which is primarily responsible for the here observed Ca^{2+} release. This conclusion may be derived by considering the effect of the H_2O_2 scavenger, catalase, which largely lowers the iron-induced burst in the cyanide-insensitive oxygen consumption. Indeed, it has previously been shown [11] and here confirmed, that iron(III)-gluconate complex undergoes redox cycling in mitochondria giving rise to a burst in O_2 consumption which for a large part may be accounted for by H_2O_2 formation (see Fig. 6).

As to the effect of this iron complex on the functional integrity of mitochondria it is conceivable that the forced Ca^{2+} cycling resulting from the iron-induced Ca^{2+} efflux unavoidably leads to mitochondrial damages. This biochemical mechanism of in vitro iron-induced release of Ca^{2+} and its possible functional implications may be of importance for the comprehension of the mitochondrial abnormalities observed in the experimental hepatic iron overload [6–8] and may give a contribution for the explanation of iron cell toxicity.

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References

1 Jacobs, A. (1980) in Iron in Biochemsitry and Medicine II (Jacobs, A. and Woorwood, M., eds.), pp. 427–459, Academic Press, London
2 Powell, L.W. (1985) in Liver and Biliary Disease (Wright, R., Millward-Sadler, G.H., Alberti, K.G.M.M. and Korgan, S., eds.), pp. 963–982, Baillere Tindall, W.B. Saunders, London
3 Bonkowsky, H.L., Healey, J.F., Sinclair, P.R., Sinclair, J.F. and Pomeroy, J.S. (1981) Biochem. J. 196, 57–64
4 Bacon, B.R., Tavill, A.S., Brittenham, G.M., Park, C.H. and Recknagel, R.O. (1985) in Free Radicals in Liver Injury (Poli, G., Cheeseman, K.H., Dianzani, M.U. and Slater, T.F., eds.), pp. 49–55, IRL Press, Oxford
5 Peters, T.J. and Seymour, C.A. (1976) Clin. Sci. Mol. Med. 50, 75–78
6 Masini, A., Trenti, T., Ventura, E., Ceccarelli-Stanzani, D. and Muscatello, U. (1984) Biochem. Biophys. Res. Commun. 124, 462–469
7 Masini, A., Ceccarelli-Stanzani, D., Trenti, T. and Ventura, E. (1984) Biochim. Biophys. Acta 802, 253–258
8 Bacon, B.R., Park, C.H., Brittenham, G.M., O'Neill, R. and Tavill, A.S. (1985) Hepatology 5, 789–797
9 Bacon, B.R., Tavill, A.S., Brittenham, G.M., Park, C.H. and Recknagel, R.O. (1983) J. Clin. Invest. 71, 429–439
10 Masini, A., Trenti, T., Ventura, E., Ceccarelli, D. and Muscatello, U. (1985) Biochem. Biophys. Res. Commun. 130, 207–213
11 Masini, A., Trenti, T., Ceccarelli-Stanzani, D. and Ventura, E. (1985) Biochim. Biophys. Acta 810, 20–26
12 Masini, A., Trenti, T., Ceccarelli-Stanzani, D. and Ventura, E. (1985) Biochim. Biophys. Acta 810, 27–32
13 Masini, A., Trenti, T., Ceccarelli, D. and Ventura, E. (1985) in Free Radicals in Liver Injury (Poli, G., Cheeseman,

K.M., Dianzani, M.U. and Slater, T.F., eds.), pp. 135–138, IRL Press, Oxford
14 Kapus, A. and Lukas, G.L. (1986) Biochem. Pharmacol. 35, 2119–2122
15 Masini, A., Ceccarelli-Stanzani, D. and Muscatello, U. (1983) Biochim. Biophys. Acta 724, 251–257
16 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 75, 1690–1694
17 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) Eur. J. Biochem. 110, 211–216
18 Crompton, M., Moser, R., Ludi, H. and Carafoli, E. (1978) Eur. J. Biochem. 82, 25–31
19 Affolter, H. and Sigel, E. (1979) Anal. Biochem. 97, 315–319
20 Botti, B., Bini, A., Calligaro, A., Meletti, E., Tomasi, A. and Vannini, V. (1986) Toxicol. Appl. Pharmacol. 83, 494–505
21 Bernardes, C.F., Pereira da Silva, L. and Vercesi, A.E. (1986) Biochim. Biophys. Acta 850, 41–48
22 Beatrice, M.C., Stiers, D.L. and Pfeiffer, D.R. (1984) J. Biol. Chem. 259, 1279–1287
23 Moore, C. (1971) Biochem. Biophys. Res. Commun. 42, 298–305
24 Nicholls, D.G. and Crompton, M. (1980) FEBS Lett. 111, 261–268
25 Lehninger, A.L., Vercesi, A. and Bababumni, E.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1680–1684
26 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1978) Proc. Natl. Acad. Sci. USA 76, 4340–4344
27 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330
28 Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) J. Biol. Chem. 257, 11558–11562
29 Bellomo, G., Martino, A., Richelmi, P., Moore, G.A., Jewell, S.A. and Orrenius, S. (1984) Eur. J. Biochem. 140, 1–6
30 Frei, B., Winterhalter, K.H. and Richter, C. (1985) J. Biol. Chem. 260, 7394–7401
31 Graf, M., Frei, B., Winterhalter, K.H. and Richter, C. (1985) Biochem. Biophys. Res. Commun. 129, 18–25
32 Frei, B. and Richter, C. (1986) FEBS Lett. 198, 99–102
33 Rugolo, M., Siliprandi, D., Siliprandi, N. and Toninello, A. (1981) Biochem. J. 200, 481–486
34 Harris, E.J., Al-Shaikhaly, H. and Baum, H. (1975) Biochem. J. 182, 455–464
35 Vercesi, A.E. (1984) Biochem. Biophys. Res. Commun. 119, 305–310
36 Masini, A., Botti, B., Ceccarelli, D., Muscatello, U. and Vannini, V. (1986) Biochim. Biophys. Acta 852, 19–24