Menadione- (2-Methyl-1,4-naphthoquinone-) Dependent Enzymatic Redox Cycling and Calcium Release by Mitochondria†

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ABSTRACT: The results presented in this paper reveal the existence of three distinct menadione (2-methyl-1,4-naphthoquinone) reductases in mitochondria: NAD(P)H:(quinone-acceptor) oxidoreductase (D,T-diaphorase), NADPH:(quinone-acceptor) oxidoreductase, and NADH:(quinone-acceptor) oxidoreductase. All three enzymes reduce menadione in a two-electron step directly to the hydroquinone form. NADH-ubiquinone oxidoreductase (NADH dehydrogenase) and NAD(P)H azoreductase do not participate significantly in menadione reduction. In mitochondrial extracts, the menadione-induced NAD(P)H oxidation occurs beyond stoichiometric reduction of the quinone and is accompanied by O_2 consumption. Benzoquinone is reduced more rapidly than menadione but does not undergo redox cycling. In intact mitochondria, menadione triggers oxidation of intramitochondrial pyridine nucleotides, cyanide-insensitive O_2 consumption, and a transient decrease of $\Delta\psi$. In the presence of intramitochondrial Ca^{2+}, the meandione-induced oxidation of pyridine nucleotides is accompanied by their hydrolysis, and Ca^{2+} is released from mitochondria. The menadione-induced Ca^{2+} release leaves mitochondria intact, provided excessive Ca^{2+} cycling is prevented. In both selenium-deficient and selenium-adequate mitochondria, menadione is equally effective in inducing oxidation of pyridine nucleotides and Ca^{2+} release. Thus, menadione-induced Ca^{2+} release is mediated predominantly by enzymatic two-electron reduction of menadione, and not by H_2O_2 generated by menadione-dependent redox cycling. Our findings argue against D,T-diaphorase being a control device that prevents quinone-dependent oxygen toxicity in mitochondria.

Quinones and their phenol precursors are widespread in the human diet (Ames, 1983). Quinones are quite toxic as they can act as electrophiles or accept a single electron (Moore & Czerniak, 1981) to yield the semiquinone radical, which can react directly with DNA (Sawahata & Neal, 1983) or participate in a redox cycle of superoxide radical (O_2^-) generation by transfer of electrons to O_2 (Kappus & Sies, 1981). O_2^- and its metabolic product H_2O_2 may form hydroxyl radicals ($OH^\bullet$) which are toxic due to their extreme and indiscriminatory reactivity. Alternatively, quinones may be reduced by a two-electron transfer to the more stable hydroquinone. This reduction can be catalyzed by the enzyme D,T-diaphorase. In microsomes, D,T-diaphorase has been shown to compete with the one-electron-transfer enzyme NADPH-cytochrome P-450 reductase and to diminish formation of semiquinones (Lind et al., 1982). Therefore, it has been proposed (Lind et al., 1982) that D,T-diaphorase constitutes a cellular device controlling semiquinone and superoxide radical formation and thereby quinone toxicity.

Besides D,T-diaphorase and NADPH-cytochrome P-450 reductase, the isolated enzyme NADH dehydrogenase can catalyze reduction of menadione (Iyanagi & Yamazaki, 1970). In addition, two diaphorases using NADH and NADPH as electron donors have been identified in hog liver cytoplasm (Koli et al., 1969). Their activities were characterized by use of the inhibitors dicoumarol and 2,4-dinitrophenol and by their reactivity toward various quinoid substrates. The presence of these two diaphorases in mitochondria was not reported. Actually, little is known about the metabolism of menadione in mitochondria. Bellomo et al. (1982) have shown that menadione impairs the ability of mitochondria to take up and retain Ca^{2+}. They suggested D,T-diaphorase and NADH dehydrogenase as the enzymes catalyzing reduction of menadione in mitochondria. However, no experimental support for this suggestion was given. Furthermore, it was not clarified whether in mitochondria menadione undergoes redox cycling and how menadione ultimately triggers Ca^{2+} release.

Many substances induce loss of mitochondrial Ca^{2+} simply by causing nonspecific damage. On the other hand, several chemically distinct compounds such as acetoacetate, *tert*-butyl hydroperoxide, or alloxan are known to induce Ca^{2+} release from intact rat liver mitochondria and by a mechanism linked to oxidation and hydrolysis of intramitochondrial pyridine nucleotides (Lehninger et al., 1978; Loetscher et al., 1979, 1980a; Frei et al., 1985a; Graf et al., 1985). Here we show that menadione does not impair the integrity of mitochondria but stimulates Ca^{2+} release by the pyridine nucleotide linked mechanism. Our data suggest that in mitochondria (i) D,T-diaphorase, NADPH:(quinone-acceptor) oxidoreductase, and NADH:(quinone-acceptor) oxidoreductase, but not NADH dehydrogenase or NAD(P)H azoreductase, catalyze reduction of menadione, (ii) menadione is reduced in a two-electron step to the hydroquinone, (iii) the hydroquinone autoxidizes, (iv) oxidation of intramitochondrial pyridine nucleotides is predominantly afforded by the above-mentioned three enzymes and not by redox cycling derived H_2O_2 via glutathione peroxidase, glutathione reductase, and the energy-linked transhydrogenase, and (v) a possibly observed damage induced by menadione is caused by excessive Ca^{2+} cycling but not by indiscriminately reactive oxygen species which are formed during redox cycling of menadione.

Experimental Procedures

Isolation of Mitochondria. Female Wistar rats (180–250 g) were fasted overnight and killed by decapitation. Liver mitochondria were isolated by the conventional differential

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Table I: Menadione- and *p*-Benzoquinone-Dependent NAD(P)H Oxidation and Oxygen Consumption by Mitochondrial Triton X-100 Extracts[a]

additions	NAD(P)H oxidation		O_2 consumption	
	nmol min^{-1} mg^{-1}	%	nmol min^{-1} mg^{-1}	%
NADPH and				
menadione	130	100	32	100
menadione and dicoumarol	3	2	1	3
menadione and 0.16 mM 2,4-dinitrophenol	60	46	ND	ND
menadione and 1 mM 2,4-dinitrophenol	ND	ND	14	44
p-benzoquinone	135[b]	104	0	0
NADH and				
menadione	68	100	24	100
menadione and dicoumarol	10	15	5	21
menadione and 0.16 mM 2,4-dinitrophenol	29	43	ND	ND
menadione and 1 mM 2,4-dinitrophenol	ND	ND	7	29
p-benzoquinone	330[b]	485	0	0

[a] Pyridine nucleotide oxidation and O_2 consumption induced by 50 μM menadione or 50 μM *p*-benzoquinone were measured as described under Experimental Procedures. Initial reaction rates are given. The reactions proceeded linearly for at least 1 min. Dicoumarol was used at a concentration of 30 μM. ND, not determined. [b] Corrected for nonenzymatic oxidation.

centrifugation method (Klingenberg & Slenczka, 1959) using 210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[1] pH 7.4 (MSH buffer), and 1 mM EDTA as isolation medium. Mitochondria were washed twice in MSH buffer. To obtain mitoplasts, mitochondria were treated for 15 min at 4 °C with digitonin (0.12 mg/mg of protein) and washed twice in MSH buffer. Submitochondrial particles were obtained by exposure of mitoplasts at 25 mg of protein/mL to sonic oscillation for 1.5 min (Branson B 30 sonifier). Larger particles were removed by a 10-min centrifugation at 10000*g*. Submitochondrial particles were collected as a pellet after centrifugation for 35 min at 100000*g*. The supernatant of this ultracentrifugation is referred to as matrix fraction. The protein content of the final suspensions was determined by the biuret method with bovine serum albumin as a standard.

Preparation of Extracts. Mitochondria or mitoplasts, 26 mg of protein/mL, were incubated with 1% (w/w) Triton X-100 for 10 min at 4 °C and centrifuged at 10000*g* for 10 min. The supernatant was used for determination of menadione-induced oxidation of NAD(P)H and O_2 consumption (see below).

Determination of Pyridine Nucleotides. NAD(P)H oxidation was followed at 340 nm by using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The reaction mixture contained, in a final volume of 1 mL, 0.14 mM NAD(P)H, 50 μM menadione, and 0.3 mg of mitochondrial protein in MSH buffer. When extracts were used, the final Triton X-100 concentration was 0.013% (w/w). Intramitochondrial pyridine nucleotides were determined in an Aminco DW 2A spectrophotometer at 340–370 nm.

Determination of Oxygen Consumption. Mitochondrial extracts (0.5 mg of protein) were investigated in a final volume of 1 mL at 25 °C in MSH buffer containing 0.14 mM NAD(P)H. The reaction was started with 50 μM menadione. Mitochondria were incubated under standard conditions (see below). O_2 consumption was determined with a Clark type electrode.

Standard Incubation Procedure for Mitochondria. Mitochondria, 2 mg of protein/mL, were incubated at 25 °C in 3 mL of MSH buffer containing 5 μM rotenone and 2.5 mM potassium succinate with continuous stirring and oxygenation (except during O_2 consumption measurements). When appropriate, mitochondria were depleted of or loaded with Ca^{2+} as described (Frei et al., 1985a). The final loads given under Results are the sum of endogenous and added Ca^{2+}. At time zero, menadione was added.

Determination of Ca^{2+} Uptake and Release by Mitochondria. Ca^{2+} movements across the inner mitochondrial membrane were followed in the presence of 50 μM of purified arsenazo III by dual wavelength spectrophotometry at 685–675 nm.

Selenium Deficiency. Selenium-deficient mitochondria were isolated from rats fed on a selenium-deficient diet for 5–7 weeks (Loetscher et al., 1979). Glutathione peroxidase activity of these mitochondria was determined as described (Loetscher et al., 1979).

Determination of Nicotinamide Release. Pyridine nucleotides were labeled in vivo (Loetscher et al., 1980a). Mitochondria from the livers of these animals were incubated according to the standard procedure. To determine the release of nicotinamide, 150-μL aliquots were withdrawn at the times indicated, filtered through Millipore filters (0.45-μm pore size), and rinsed twice with 150 μL of cold MSH buffer. The radioactivity remaining on the filters was determined in a liquid scintillation counter.

Determination of $\Delta\psi$ was done as reported (Loetscher et al., 1980b). Mitochondria were incubated under standard conditions in MSH buffer and 20 μM tetraphenylphosphonium (TPP^+).

Results

Mixing of equimolar amounts of menadione and NAD(P)H does not cause NAD(P)H oxidation. However, addition of menadione to mitochondria results in oxidation of intramitochondrial pyridine nucleotides (see Figure 4b). Since dicoumarol, a potent tool in investigating quinone reductases (Ernster et al., 1962; Koli et al., 1969), uncouples mitochondria, the mitochondrial enzymes involved in menadione-induced pyridine nucleotide oxidation were investigated with mitochondrial extracts obtained with Triton X-100 (Table I). Although NADH and NADPH are equally good substrates for D,T-diaphorase (Ernster et al., 1962), in mitochondrial extracts NADPH is oxidized much more rapidly than NADH. Dicoumarol, which inhibits D,T-diaphorase and NADPH:(quinone-acceptor) oxidoreductase but not NADH:(quinone-acceptor) oxidoreductase (Koli et al., 1969), inhibits the menadione-induced oxidation of NADPH completely but that of NADH only partly. These results suggest that in mito-

[1] Abbreviations: arsenazo III, 2,2′-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-diylbisazo)bis(benzenearsonic acid); $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MSH buffer, 210 mM mannitol, 70 mM sucrose, and 5 mM HEPES, pH 7.4; TPP^+, tetraphenylphosphonium.

Table II: Menadione- and *p*-Benzoquinone-Induced NAD(P)H Oxidation by Mitochondria and Their Subfractions[a]

	NADPH oxidation (nmol min^{-1} mg^{-1})	NADH oxidation (nmol min^{-1} mg^{-1})
mitochondria	2	6
Triton X-100 extract of mitochondria	129	57
mitoplasts	1	5
Triton X-100 extract of mitoplasts	143	71
matrix fraction	184	44
+dicoumarol	4	5
p-benzoquinone	ND	157[b]
submitochondrial particles	10	18[c]
+dicoumarol	0	18[c]
p-benzoquinone	ND	397[b,c]

[a] Pyridine nucleotide oxidation induced by 50 μM menadione or 50 μM *p*-benzoquinone was measured as described under Experimental Procedures. Initial reaction rates are given. The reactions proceeded linearly for at least 1 min. Dicoumarol was used at a concentration of 30 μM. ND, not determined. [b] Corrected for nonenzymatic oxidation. [c] Determined in the presence of 5 μM rotenone.

chondria besides D,T-diaphorase at least two other menadione reductases are present. The oxidation of NADPH in mitochondrial extracts is only partly inhibited by 2,4-dinitrophenol. Since D,T-diaphorase is completely inhibited by 2,4-dinitrophenol, and NADPH:(quinone-acceptor) oxidoreductase is not sensitive to this substance (Koli et al., 1969), this result strongly suggests the existence of NADPH:(quinone-acceptor) oxidoreductase in mitochondria. This conclusion is supported by the finding (Table I) that 50 μM *p*-benzoquinone stimulates oxidation of NADPH only slightly [cf. Koli et al. (1969)]. The oxidation of NADH, on the other hand, is dramatically enhanced with *p*-benzoquinone (Table I). Since, compared to menadione, the activity of NADH:(quinone-acceptor) oxidoreductase is stimulated by a factor of about 24 (Koli et al., 1969) with *p*-benzoquinone as substrate, this drastic increase most probably is due to the existence of a NADH:(quinone-acceptor) oxidoreductase in mitochondria. Unlike the enzyme from hog liver cytoplasm (Koli et al., 1969), but similar to the NADH:(quinone-acceptor) oxidoreductase from *H. eutropha* (Repaske & Lizotte, 1965) the mitochondrial NADH:(quinone-acceptor) oxidoreductase is not inhibited by 2,4-dinitrophenol.

The menadione- but not the *p*-benzoquinone-induced oxidation of NADH and NADPH in mitochondrial extracts occurs beyond stoichiometry: addition of 50 μM menadione to a solution containing 140 μM NAD(P)H and 0.3 mg of mitochondrial protein results in a total oxidation of pyridine nucleotides. This observation implies reoxidation of menadione after its enzymatic reduction. Indeed, the menadione-induced oxidation of NAD(P)H in mitochondrial extracts is accompanied by O_2 consumption (Table I). In contrast to the situation in microsomes (Lind et al., 1982), in mitochondrial extracts the extent of inhibition by dicoumarol and 2,4-dinitrophenol of the menadione-induced NAD(P)H oxidation and the accompanying O_2 consumption is essentially the same. Since D,T-diaphorase catalyzes a two-electron transfer (Iyanagi & Yamazaki, 1970; Lind et al., 1982), and since its inhibition by dicoumarol and 2,4-dinitrophenol induces the same percent decrease of both NAD(P)H oxidation and O_2 consumption, we conclude that all mitochondrial enzymes significantly involved in menadione reduction are two-electron-transfer enzymes. This notion is strongly supported by the fact that *p*-benzoquinone cannot undergo redox cycling in mitochondrial extracts (Table I) or intact mitochondria (result not shown). Iyanagi and Yamazaki (1970) have shown

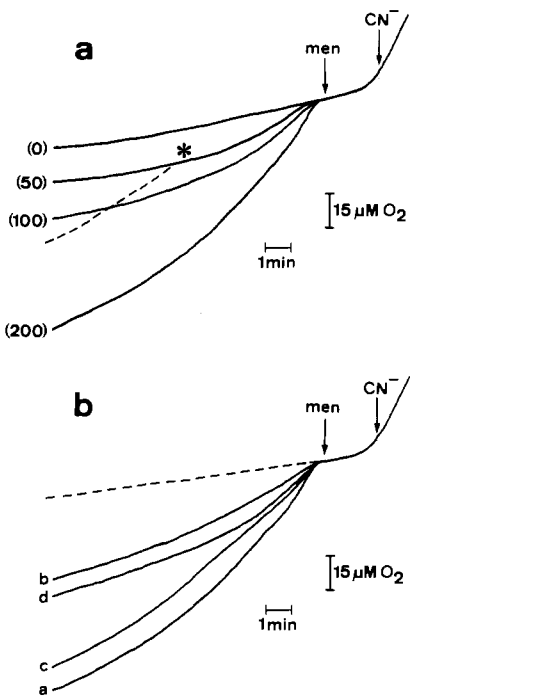

FIGURE 1: Oxygen consumption by rat liver mitochondria in the presence of cyanide and menadione. Mitochondria were incubated under standard conditions in the presence of rotenone and potassium succinate. Oxygen consumption was followed with a Clark-type electrode. Cyanide (CN^-) (200 μM) was added where indicated. (Panel a) At the *arrow*, menadione was added (number in parentheses, μM). The *dashed line* was obtained upon a second dose of 50 μM menadione, added at the *asterisk*. (Panel b) At the *arrow*, 200 μM menadione was added. (Curve a) no further addition was made; (curve b) 30 μM dicoumarol was added 30 s before cyanide; (curve c) 100 μM *tert*-butyl hydroperoxide was added 2 min before cyanide. For curve d, the protein content was 1 mg/mL. The *dashed line* was obtained in the absence of menadione.

that, in contrast to D,T-diaphorase, the isolated one-electron-transfer enzyme NADH dehydrogenase induces redox cycling with *p*-benzoquinone as substrate.

The localization of the mitochondrial menadione reductases was investigated by using mitochondrial subfractions (Table II). With both mitochondria and mitoplasts, significant menadione-induced oxidation of NAD(P)H cannot be observed unless the membranes are solubilized. This indicates that all mitochondrial menadione reductases are localized inside mitochondria. Moreover, it excludes NAD(P)H oxidation by NAD(P)H azoreductase (Moreno et al., 1984) of the mitochondrial outer membrane. Menadione-induced oxidation of NADPH is catalyzed predominantly by the matrix fraction, and that of NADH at a significant rate by both matrix fraction and submitochondrial particles (Table II). The activity of submitochondrial particles is insensitive to dicoumarol and stimulated 22-fold with *p*-benzoquinone. The results of Table II taken together strongly suggest that D,T-diaphorase and NADPH:(quinone-acceptor) oxidoreductase are localized predominantly in the mitochondrial matrix and NADH:(quinone-acceptor) oxidoreductase in the mitochondrial inner membrane.

The fact that in mitochondrial extracts the menadione-induced NAD(P)H oxidation is accompanied by O_2 consumption (see Table I) led us to investigate possible redox cycling of menadione also in intact mitochondria. To this end, O_2 consumption by the respiratory chain was blocked by 200 μM cyanide, and the menadione-induced extra O_2 consumption was determined (Figure 1a). Menadione at 50–200 μM causes a dose-dependent increase in O_2 consumption. *p*-

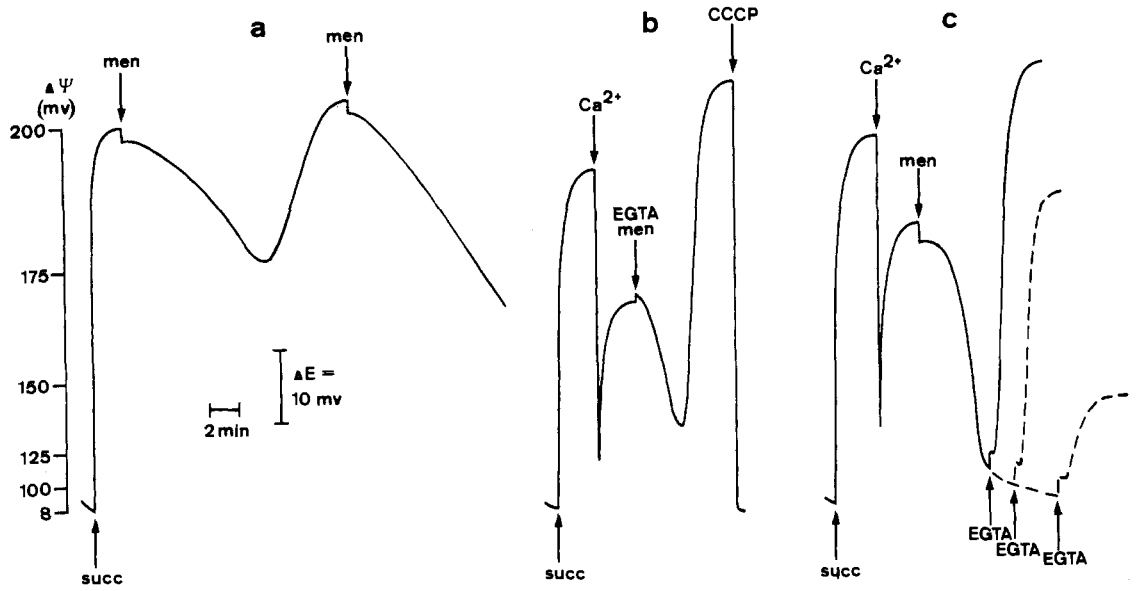

FIGURE 2: Menadione-induced changes of mitochondrial membrane potential. Mitochondria were incubated under standard conditions in 1 mL of MSH buffer containing 20 μM TPP^+. Mitochondria were depleted of Ca^{2+} with rotenone and EGTA (panel a) or loaded with 53 nmol of Ca^{2+}/mg of protein (panels b and c). At the *arrows*, the following additions were made: 2.5 mM potassium succinate (succ), Ca^{2+}, 200 μM menadione (men), 0.5 mM EGTA, and 1.3 μM carbonyl cyanide (*m*-chlorophenyl)hydrazone (CCCP). ΔE, electrode potential.

Benzoquinone at 100 μM does not induce extra O_2 consumption (result not shown). With small amounts of menadione (e.g., 50 μM) the increased O_2 consumption is clearly transient and can be evoked repeatedly (Figure 1a). In the presence of 30 μM dicoumarol (Figure 1b) much less O_2 is consumed. The extra O_2 consumption is also strongly inhibited by 1 mM 2,4-dinitrophenol (result not shown). Furthermore, a link between mitochondrial-reduced pyridine nucleotides and menadione-induced redox cycling is indicated by the observation (Figure 1b) that in mitochondria whose pyridine nucleotides are mainly oxidized due to the presence of *tert*-butyl hydroperoxide the menadione-induced redox cycling is diminished.

Reactive oxygen species generated by redox cycling of menadione are considered as the culprits of menadione toxicity (Bachur et al., 1978; Lind et al., 1982; Smith et al., 1982). Besides being mutagenic (Chesis et al., 1984), menadione disturbs cellular Ca^{2+} homeostasis by impairing the ability of mitochondria to retain Ca^{2+} (Bellomo et al., 1982; Thor et al., 1982). However, it remained unclear whether menadione triggers Ca^{2+} release from mitochondria by causing nonspecific damage or via the well established specific mechanism (Lehninger et al., 1978; Loetscher et al., 1979, 1980a,b; Frei et al., 1985a,b; Graf et al., 1985), i.e., via oxidation and hydrolysis of mitochondrial pyridine nucleotides. We therefore examined possible menadione-induced damage of mitochondria by determination of the mitochondrial transmembrane potential ($\Delta\psi$) (Figure 2). In mitochondria depleted of Ca^{2+} (Figure 2a), addition of 200 μM menadione induces a slight and transient decrease of $\Delta\psi$. A second dose of 200 μM menadione again causes $\Delta\psi$ to decrease. This time, however, no recovery of $\Delta\psi$ is seen. Also in Ca^{2+}-loaded mitochondria (Figure 2b) a transient decrease of $\Delta\psi$ is observed upon addition of menadione, provided EGTA is given simultaneously to prevent Ca^{2+} cycling. When menadione is added to Ca^{2+}-loaded mitochondria in the absence of EGTA (Figure 2c), $\Delta\psi$ decreases drastically. Nevertheless, mitochondria are still able to restore $\Delta\psi$ when EGTA is added a few minutes after menadione. These data show that 200 μM menadione alone as well as the menadione-induced Ca^{2+} release leaves mitochondria intact, provided excessive Ca^{2+} cycling is prevented.

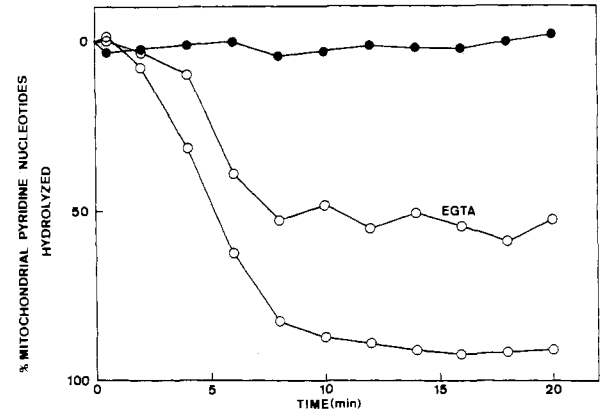

FIGURE 3: Menadione-induced hydrolysis of pyridine nucleotides in rat liver mitochondria. Intramitochondrial pyridine nucleotides were labeled in vivo at the nicotinamide moiety. Incubation of mitochondria was according to the standard procedure. Mitochondria were depleted of Ca^{2+} with rotenone and EGTA (●) or loaded with 53 nmol of Ca^{2+}/mg of protein (○). The release of nicotinamide from mitochondria was measured with the Millipore filtration technique. At zero time, 50 μM menadione was added. Where indicated, 0.5 mM EGTA was added together with menadione.

For the operation of the specific Ca^{2+} release mechanism, oxidation of pyridine nucleotides is a necessary but not sufficient event. Rather, only when hydrolysis of oxidized pyridine nucleotides takes place is Ca^{2+} release observed (Loetscher et al., 1980a; Frei et al., 1985a,b; Graf et al., 1985). To examine a possible stimulation of this specific pathway, we investigated whether menadione can trigger hydrolysis of intramitochondrial pyridine nucleotides. For that purpose, we labeled intramitochondrial pyridine nucleotides in vivo at the nicotinamide moiety and determined possible menadione-induced release of nicotinamide from mitochondria, indicating pyridine nucleotide hydrolysis (Loetscher et al., 1980a). In the absence of intramitochondrial Ca^{2+}, 50 μM menadione does not induce loss of radioactivity from mitochondria (Figure 3). In contrast, virtually complete release of radioactivity is seen from mitochondria loaded with 53 nmol of Ca^{2+}/mg of protein. When EGTA is added together with menadione, i.e., under noncycling conditions, the amount of radioactivity re-

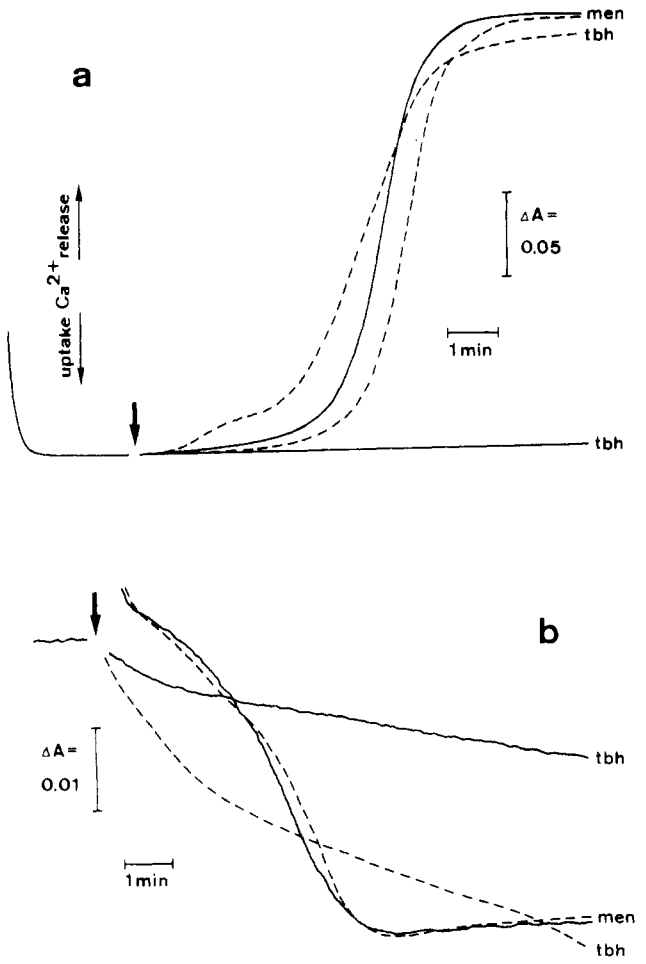

FIGURE 4: Influence of selenium deficiency on menadione-induced Ca^{2+} release and pyridine nucleotide oxidation in rat liver mitochondria. Mitochondria were incubated according to the standard procedure and loaded with 60 nmol of Ca^{2+}/mg of protein. At the *arrow*, 100 μM menadione (men) or 100 μM *tert*-butyl hydroperoxide (tbh) were added. (—) Selenium-deficient mitochondria; (---) selenium-adequate mitochondria. (Panel a) Ca^{2+} movements were monitored at 685–675 nm with the aid of 50 μM arsenazo III. (Panel b) the redox level of mitochondrial pyridine nucleotides was followed spectrophotometrically at 340–370 nm.

leased is diminished (Figure 3). The continuous presence of intramitochondrial Ca^{2+} therefore may be a prerequisite for pyridine nucleotide hydrolysis.

It is evident from the above results that menadione stimulates the specific Ca^{2+} release pathway. Still, H_2O_2 derived from redox cycling could, at least in part, be responsible for this effect. It is well-known that reduction of hydroperoxides via glutathione peroxidase, glutathione reductase, and the energy-linked transhydrogenase causes oxidation of intramitochondrial pyridine nucleotides and thereby can stimulate Ca^{2+} release (Loetscher et al., 1979, 1980a; Frei et al., 1985b). Hydroperoxide reduction by glutathione peroxidase is prevented by selenium deficiency (Loetscher et al., 1980a). To evaluate the contribution of H_2O_2 to the menadione-induced Ca^{2+} release, we therefore isolated mitochondria from selenium-adequate and selenium-deficient mitochondria. Menadione is about equally effective in inducing pyridine nucleotide oxidation and Ca^{2+} release in both mitochondrial preparations, whereas the effects of the control substance *tert*-butyl hydroperoxide are strongly prevented by selenium-deficiency (Figure 4). These results are in line with the observation by Bellomo et al. (1982) that depletion of mitochondrial glutathione by diethyl maleate does not affect the Ca^{2+} fluxes induced by menadione.

Discussion

Menadione causes pyridine nucleotide oxidation and cyanide-insensitive oxygen consumption in mitochondria and in mitochondrial extracts supplemented with reduced pyridine nucleotides. The pattern of inhibition obtained with dicoumarol and 2,4-dinitrophenol of menadione-induced pyridine nucleotide oxidation and concomitant oxygen consumption, and the stimulation by *p*-benzoquinone of pyridine nucleotide oxidation without oxygen consumption allow the conclusions that in mitochondria (i) menadione is reduced in a two-electron step by the three enzymes D,T-diaphorase, NADPH:(quinone-acceptor) oxidoreductase, and NADH:(quinone-acceptor) oxidoreductase, (ii) NADH dehydrogenase is not involved in menadione reduction, and (iii) reduced menadione autoxidizes.

According to Lind et al. (1982) D,T-diaphorase serves as a cellular control device against quinone toxicity. This proposal is supported by the finding that D,T-diaphorase reduces quinones by a two-electron transfer to rather stable hydroquinones (Iyanagi & Yamazaki, 1970; Lind et al., 1982). Most other NAD(P)H-oxidizing flavoproteins are one-electron-transfer enzymes (Iyanagi & Yamazaki, 1970) forming semiquinones which autoxidize more readily than hydroquinones with the formation of O_2^-. Indeed, in rat liver microsomes D,T-diaphorase competes successfully with NADPH-cytochrome P-450 reductase for menadione. As a consequence, when D,T-diaphorase is active, redox cycling is low. In contrast, when D,T-diaphorase is inhibited by dicoumarol, NADPH oxidation induced by menadione continues beyond stoichiometry, and redox cycling is increased (Lind et al., 1982). Similarly, dicoumarol stimulates the menadione-dependent redox cycling in hepatocytes (Thor et al., 1982). In contrast, in intact mitochondria or in mitochondrial extracts menadione is reduced exclusively by two-electron-transfer enzymes. Autoxidation therefore starts from completely reduced menadione. It can be calculated from the data shown in Table I that in mitochondria about 40% of the menadione-dependent pyridine nucleotide oxidation and redox cycling is catalyzed by D,T-diaphorase. Therefore, in mitochondria D,T-diaphorase cannot be considered a control device against quinone-dependent oxygen toxicity as in microsomes where competition between one- and two-electron-transfer enzymes for menadione occurs.

Subfractionation of mitochondria reveals the presence of D,T-diaphorase and NADPH:(quinone-acceptor) oxidoreductase in the mitochondrial matrix and of NADH:(quinone-acceptor) oxidoreductase in the mitochondrial inner membrane. It also excludes NAD(P)H azoreductase to be significantly involved in mitochondrial menadione reduction.

The observations that in solubilized mitochondria both pyridine nucleotide oxidation and oxygen consumption induced by menadione occur beyond stoichiometry imply extensive regeneration of menadione by autoxidation after its enzymatic reduction. However, in the presence of succinate, rotenone, and nonlimiting amounts of oxygen, in intact mitochondria menadione-induced extra oxygen consumption and changes in $\Delta\psi$ are transient and can be evoked repeatedly (see Figures 1a and 2a). This suggests further metabolism of menadione, possibly conjugation, provided mitochondria are intact.

Reactive oxygen species generated by menadione-dependent redox cycling neither contribute significantly to the oxidation of intramitochondrial pyridine nucleotides nor impair the integrity of mitochondria. Rather, menadione triggers Ca^{2+} release due to pyridine nucleotide oxidation via the three mitochondrial menadione reductases indentified in this paper. As shown by menadione-induced pyridine nucleotide hydrolysis

and intactness of mitochondria during and after Ca^{2+} release, menadione stimulates the specific Ca^{2+} release pathway. Ca^{2+} release, therefore, proceeds via the Ca^{2+}/proton antiport (Fiskum & Lehninger, 1980) and not by reversal of the Ca^{2+} uptake route. Only when $\Delta\psi$ reaches values of 130 mV or lower, due to Ca^{2+} cycling, should such a reversal of the uptake route be considered (Nicholls, 1978).

In conclusion, menadione induces Ca^{2+} release from intact mitochondria via the specific pyridine nucleotide linked pathway. Reactive oxygen species produced by redox cycling of menadione in mitochondria do not significantly contribute to this Ca^{2+} release. Nevertheless, menadione may still induce damage to mitochondria and the intact cell due to excessive mitochondrial Ca^{2+} cycling and disturbance of cellular Ca^{2+} homeostasis.

Registry No. NADPH, 53-57-6; NADH, 58-68-4; EC 1.6.99.2, 9032-20-6; EC 1.6.99.6, 37256-37-4; EC 1.6.99.5, 37256-36-3; O_2, 7782-44-7; Ca, 7440-70-2; menadione, 58-27-5; *p*-benzoquinone, 106-51-4; menadione hydroquinone, 5022-70-8.

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