THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 20, Issue of October 25, pp. 12419-12425, 1982
Printed in U.S.A.

The Metabolism of Menadione (2-Methyl-1,4-naphthoquinone) by Isolated Hepatocytes

A STUDY OF THE IMPLICATIONS OF OXIDATIVE STRESS IN INTACT CELLS*

(Received for publication, March 11, 1982)

Hjördis Thor‡, Martyn T. Smith§, Pia Hartzell‡, Giorgio Bellomo‡¶, Sarah A. Jewell‡, and Sten Orrenius‡

From the ‡Department of Forensic Medicine, Karolinska Institutet, S-104 01 Stockholm, Sweden, and the §Toxicology Unit, Department of Pharmacology, The School of Pharmacy, London WC1N 1AX, United Kingdom

The cytotoxic effects of many quinones are thought to be mediated through their one-electron reduction to semiquinone radicals, which subsequently enter redox cycles with molecular oxygen to produce active oxygen species and oxidative stress. The two-electron reduction of quinones to diols, mediated by DT-diaphorase (NAD(P)H: (quinone-acceptor) oxidoreductase), may therefore represent a detoxifying pathway which protects the cell from the formation of these reactive intermediates.

By using menadione (2-methyl-1,4-naphthoquinone) and isolated hepatocytes, the relative contribution of the two pathways to quinone metabolism has been studied and a protective role for DT-diaphorase demonstrated. Moreover, in the presence of cytotoxic concentrations of menadione rapid changes in intracellular thiol and Ca^{2+} homeostasis were observed. These were associated with alterations in the surface structure of the hepatocytes which may be an early indication of cytotoxicity.

Quinones are widely distributed in nature and many clinically important antitumor drugs contain the quinone nucleus (1). They form an important group of substrates for flavoenzymes and can undergo either two-electron reduction to the hydroquinone or one-electron reduction to the semiquinone radical (2). The antitumor and cytotoxic effects of quinonoid drugs are thought to be mediated through their one-electron reduction to semiquinone radicals (3). Most semiquinones rapidly reduce dioxygen to form superoxide anion radical ($O_2^{\cdot-}$) and thus regenerate the quinone (4). Quinones may, therefore, enter flavoprotein-catalyzed redox cycles with dioxygen which result in the formation of large amounts of $O_2^{\cdot-}$ and the oxidation of reduced pyridine nucleotides.

The enzymatic or spontaneous dismutation of $O_2^{\cdot-}$ yields H_2O_2 and O_2 (5). $O_2^{\cdot-}$ and H_2O_2 can react together, in a process catalyzed by certain metal ions, to form even more deleterious oxygen species such as the hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1\Delta g$ O_2) (6–8). The flavoprotein-catalyzed redox cycling of quinones in cells would, therefore, quickly lead to conditions of oxidative stress via the oxidation of reduced pyridine nucleotides and the formation of active oxygen species, capable of inflicting damage by processes such as lipid peroxidation (9). The redox cycling of quinonoid drugs and other related compounds has, therefore, been widely implicated as a mechanism for their cytotoxicity (8, 9).

Relatively little is known, however, about the metabolism of quinonoid drugs in cells, or the factors which govern the formation of semiquinone radicals and toxic oxygen species *in vivo*. For example, the flavoprotein (NAD(P)H: (quinone-acceptor) oxidoreductase, also known as DT-diaphorase (10), catalyzes the two-electron reduction of quinones to hydroquinones without the formation of semiquinone radical intermediates (2, 10), whereas NADPH-cytochrome P-450 reductase and NADH-ubiquinone oxidoreductase catalyze the one-electron reduction of quinones to semiquinone radicals (2, 11). As first postulated by Ernster and associates (12), DT-diaphorase may, therefore, protect cells against the oxidative stress induced by quinonoid drugs by competing with the single electron reduction pathways.

In the present investigation this potentially protective role of DT-diaphorase and other factors relevant to the metabolism and cytotoxicity of quinonoid drugs have been studied using menadione (2-methyl-1,4-naphthoquinone) and freshly isolated rat hepatocytes as the experimental model. Pretreatment of the rats with either phenobarbital or 3-methylcholanthrene facilitated the alteration of specific enzyme activities under investigation. For example, phenobarbital administration is known to induce hepatic NADPH-cytochrome P-450 reductase (13) but has little effect on DT-diaphorase, whereas 3-MC[1] pretreatment increases the activity of hepatic cytosolic DT-diaphorase (14) but has little or no effect on NADPH-cytochrome P-450 reductase (13). It has, therefore, been possible to study the relative roles of DT-diaphorase and NADPH-cytochrome P-450 reductase in regulating the metabolism and cytotoxicity of menadione in isolated hepatocytes. The results obtained clearly show a protective role for DT-diaphorase in preventing menadione cytotoxicity and demonstrate the importance of competition between activating and detoxifying pathways in regulating the oxidative stress caused by quinonoid drugs. Moreover, it has been possible using techniques recently developed in our laboratory (15) to show that one of the early events in menadione-induced cytotoxicity is a change in surface structure of the isolated hepatocytes which appears to be caused by alterations in intracellular thiol and Ca^{2+} homeostasis.

* This work was supported by grants from the Swedish Medical Research Council (Project 03X-2471), the Swedish Council for Planning and Coordination of Research, and the Nobel Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address, Clinica Medica II[a], University of Pavia, Pavia, Italy.

[1] The abbreviations used are: 3-MC, 3-methylcholanthrene; GSH, glutathione, reduced form.

EXPERIMENTAL PROCEDURES

Materials—Collagenase (Grade II) and carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone were obtained from Boehringer-Mannheim GmbH, Mannheim, FRG. Horse heart cytochrome *c*, antimycin A, superoxide dismutase, dicoumarol, menadione, arsenazo III, bovine serum albumin (Fraction V), NADH, NADPH, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were obtained from Sigma. The Ca^{2+} ionophore A23187 was from Calbiochem-Behring and Percoll was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Acetylated cytochrome *c* was prepared by the method of Azzi *et al.* (16).

Animals and Pretreatments—Male Sprague-Dawley rats (200–220 g) were used for all experiments and allowed food and water *ad libitum*. Some rats were given phenobarbital (80 mg/kg of sodium phenobarbital i.p.) for 5 days and others a single i.p. injection of 3-MC (50 mg/kg) as a 5 mg/ml solution in corn oil 40 h prior to use.

Hepatocyte Isolation and Incubation—Hepatocytes were isolated by collagenase perfusion of the liver as previously described (17). The yield of each preparation was 2–4 × 10^8 cells/liver, and immediately after isolation the hepatocytes excluded both trypan blue and NADH (90–100%). Cell viability was determined during the course of the experiments by the exclusion of trypan blue and by the NADH penetration assay (17). Krebs-Henseleit buffer, pH 7.4, containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was used for the incubation. The hepatocytes were incubated at 10^6 cells/ml in rotating round-bottom flasks as previously described (17). Menadione was added in 10 μl of dimethyl sulfoxide.

Preparation of Subcellular Fractions—The microsomal and cytosolic fractions of control rat liver were isolated as described by Ernster *et al.* (18). The subcellular fractions were incubated in 100 mM Tris-HCl buffer, pH 7.4, containing 50 mM KCl at a protein concentration of 1 mg/ml.

Biochemical Assays—Oxygen consumption was measured polarographically at 37 °C using a 2-ml water-jacketed cell fitted with a Clark oxygen electrode. Antimycin A (25 μM) was used to inhibit mitochondrial oxygen uptake during the oxygen consumption measurements in isolated hepatocytes.

Superoxide anion production was measured as the reduction of acetylated cytochrome *c* using the wavelength pair 550–540 nm (16, 19). Addition of superoxide dismutase (0.2 mg/ml) enabled the background reduction rate to be subtracted. Acetylated cytochrome *c* (0.2 mg/ml) was added prior to the addition of substrate. When subcellular fractions were used, NADPH or NADH (1 mM) was also included in the incubation.

NADPH and NADH oxidation by the subcellular fractions was measured at 340 nm using incubation conditions identical to those described above in the absence of acetylated cytochrome *c*. DT-diaphorase was assayed in the microsomal and cytosolic fractions of rat liver using NADPH oxidation at 340 nm as previously described (10). The background rate of NADPH oxidation measured in the presence of 30 μM dicoumarol was subtracted from the total rate observed. Hepatocyte GSH level was measured as acid-soluble thiols, using the colorimetric assay of Saville (20). $NAD(P)^+$ and NAD(P)H concentrations were assayed by the spectrophotometric method described in Ref. 21. Protein was determined by the method of Lowry *et al.* (22).

Ca^{2+} Compartmentation Measurements—For technical reasons (15), hepatocytes isolated from untreated rats were incubated as described above, but at a cell concentration of 6 × 10^6 cells/ml, for the measurement of intracellular Ca^{2+} distribution. The hepatocytes were separated from the Ca^{2+}-containing Krebs-Henseleit buffer by rapid centrifugation through a suspension of Percoll in Ca^{2+}- and Mg^{2+}-free Hank's solution (23) (final density: 1.06 g/ml) (15). They were then quickly resuspended in the modified Hank's medium and separated into two parts; one part was used for Ca^{2+} measurement and the other for counting the number of cells present and for assaying cell viability.

Intracellular Ca^{2+} distribution was determined by dual wavelength spectrophotometry using purified arsenazo III (2, 2'-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo)bis-(benzene arsonic acid) and the wavelength pair 685–675 nm (15, 23, 24). The Ca^{2+} releasable by carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone is thought to represent the mitochondrial Ca^{2+} pool and Ca^{2+} released by A23187, following carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone release, the extramitochondrial Ca^{2+} pool (15, 23).

Scanning Electron Microscopy—Samples were processed by standard procedures, involving glutaraldehyde and osmium fixation followed by critical point drying. A Jeol model JSM35 scanning electron microscope was used to visualize and photograph the samples. A large number of hepatocytes were examined and representative cells showing a typical morphology were photographed.

RESULTS

Effect of Dicoumarol on Menadione-stimulated Oxygen Uptake by Isolated Hepatocytes—The addition of micromolar concentrations of menadione to suspensions of isolated hepatocytes greatly stimulated oxygen consumption in the presence of antimycin A (Fig. 1). This increase occurred to approximately the same extent in hepatocytes from phenobarbital- and 3-MC-treated rats. However, concentrations of menadione less than 15 μM were without effect on oxygen uptake by hepatocytes from 3-MC-treated rats, but caused up to a 50% stimulation of oxygen consumption by hepatocytes from phenobarbital-treated rats. Fig. 1 also shows the marked effect of adding dicoumarol (30 μM), prior to menadione addition, on menadione-stimulated oxygen uptake. The presence of dicoumarol produced a left shift in the dose-response curve (Fig. 1), greatly stimulating antimycin A-independent oxygen consumption by hepatocytes isolated from both phenobarbital- and 3-MC-treated rats. The effect of dicoumarol was, however, slightly larger in hepatocytes from 3-MC-treated than from phenobarbital-treated rats (Fig. 1). At menadione concentrations greater than 50 μM, prior addition of dicoumarol was without effect. Dicoumarol addition also had no effect upon antimycin A-independent oxygen uptake by hepatocytes in the absence of menadione (Fig. 1). Taken together these results suggest that inhibition of DT-diaphorase-mediated metabolism of menadione in hepatocytes by dicoumarol increases the availability of menadione for single electron reduction and redox cycling resulting in enhanced oxygen consumption.

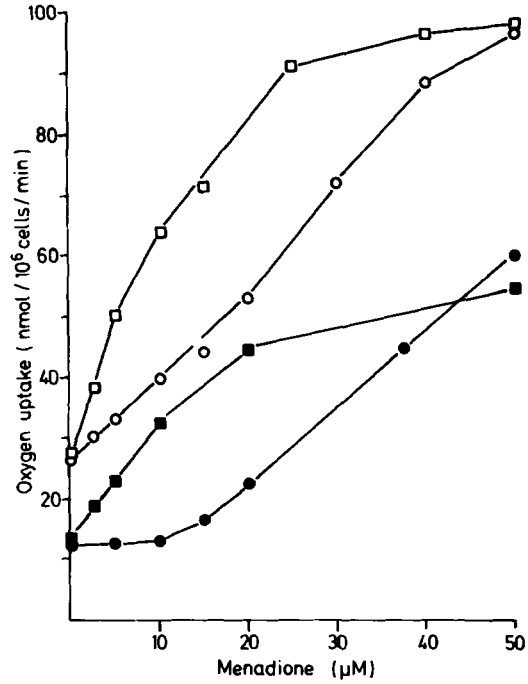

FIG. 1. **Effects of menadione and dicoumarol on antimycin A-independent O_2 uptake by hepatocytes from phenobarbital- and 3-MC-treated rats.** The O_2 consumption by hepatocytes isolated from either phenobarbital (○)- or 3-MC (●)-pretreated rats was measured polarographically in the presence of 25 μM antimycin A and varying concentrations of menadione. The effect of adding 30 μM dicoumarol prior to the addition of menadione is also shown: □, phenobarbital cells; ■, 3-MC cells. The mean values of at least four separate experiments are shown.

Superoxide Formation during the Metabolism of Menadione in Isolated Hepatocytes—Fig. 2 shows that hepatocytes isolated from phenobarbital-treated rats did not on their own liberate significant quantities of O_2^- into the medium, as measured by the reduction of acetylated cytochrome *c*. When menadione was added to the incubation medium, however, there was a marked increase in the rate of reduction of extracellular acetylated cytochrome *c* (Fig. 2). A large proportion of this menadione-dependent cytochrome *c* reduction was inhibited by addition of 0.2 mg/ml of superoxide dismutase, showing that it was due to O_2^- release from the hepatocytes. Interestingly, a significant proportion of the menadione-dependent reduction of acetylated cytochrome *c* was resistant to superoxide dismutase inhibition. Since the fully reduced form of menadione, 2-methyl-1,4-naphthohydroquinone (menadiol), is capable of reducing artificial electron acceptors such as acetylated cytochrome *c* (10–14), the reduction of cytochrome *c* observed in the presence of added superoxide dismutase may reflect the release of menadiol from the hepatocytes. The use of superoxide dismutase and dicoumarol in combination almost completely inhibited the reduction of cytochrome *c* (Fig. 2) showing that menadiol and O_2^- were the only substances reducing cytochrome *c* in this system. Dicoumarol alone did not increase the menadione-dependent release of O_2^- from hepatocytes (Fig. 2), as one would have expected from the data shown in Fig. 1. This apparently contradictory result is discussed in detail later in this report.

Superoxide Production during the Metabolism of Menadione by Isolated Subcellular Fractions—In the presence of 10 μM menadione and either NADH or NADPH both the microsomal and cytosolic fractions of rat liver catalyzed the rapid reduction of added acetylated cytochrome *c* (Fig. 3). The majority of the cytochrome *c* reduction produced by the microsomal fraction could be attributed to the formation of O_2^-, since it was prevented by the addition of superoxide dismutase. NADPH-cytochrome P-450 reductase also appears to be a more effective catalyst of menadione-dependent O_2^- formation than NADH-cytochrome b_5 reductase, since the activity obtained with NADPH was much higher than with NADH (Fig. 3). In contrast to the findings with the microsomal fraction, where superoxide dismutase addition caused an almost complete inhibition of cytochrome *c* reduction, the menadione-dependent reduction of acetylated cytochrome *c* catalyzed by the cytosolic fraction was unaffected by addition of superoxide dismutase (Fig. 3), but was almost completely inhibited by dicoumarol (Fig. 3). These results confirm that cytosolic DT-diaphorase reduces menadione to the relatively stable hydroquinone, menadiol, without the formation of O_2^-. Some DT-diaphorase is also present in the microsomal fraction and converts menadione to menadiol by the same two-electron transfer process without forming O_2^- (Fig. 3).

Depletion of Glutathione during the Metabolism of Menadione in Isolated Hepatocytes—A marked decrease in intracellular GSH level occurred independently of measurable changes in the pyridine nucleotide redox state when hepatocytes isolated from phenobarbital-treated rats were incubated with menadione concentrations greater than 35 μM (Fig. 4).

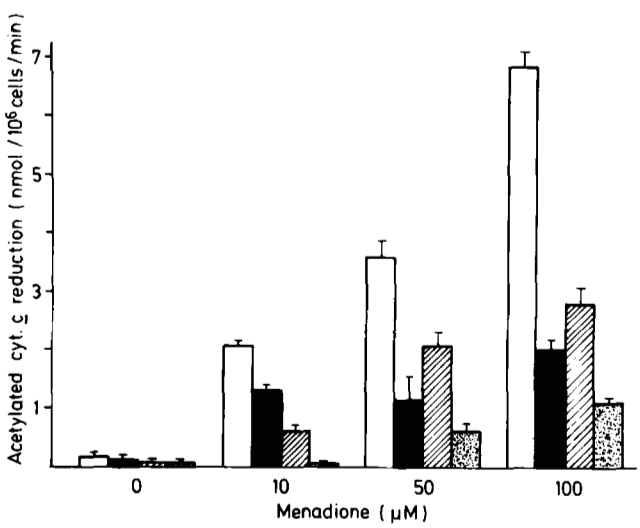

FIG. 2. **Effect of added superoxide dismutase and dicoumarol on the rate of reduction of extracellular acetylated cytochrome *c* during menadione metabolism in isolated hepatocytes.** Hepatocytes isolated from phenobarbital-treated rats were incubated (10^6 cells/ml) in Krebs-Henseleit buffer, pH 7.4, with different concentrations of menadione in the presence of acetylated cytochrome *c* (0.2 mg/ml). Dicoumarol (30 μM) (*solid bar*) or superoxide dismutase (0.2 mg/ml) (*striped bar*) was added either separately, or in combination (*speckled bar*), to test the specificity of the measurements. Values are expressed as the mean ± S.E. of three to five experiments.

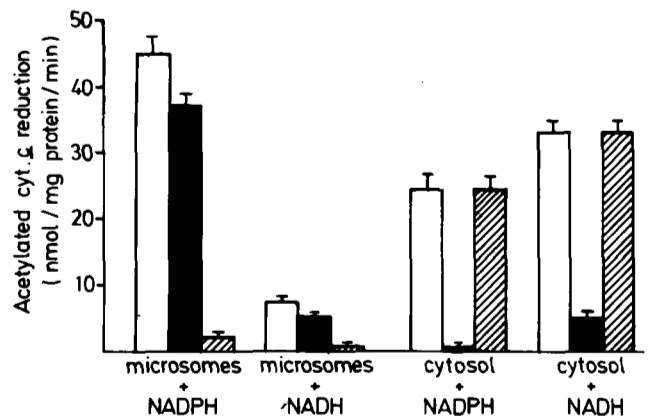

FIG. 3. **Effect of added superoxide dismutase and dicoumarol on acetylated cytochrome *c* reduction during menadione metabolism by different subcellular fractions, isolated from phenobarbital-treated rats.** The subcellular fractions (1 mg of protein/ml) were incubated with 10 μM menadione in 0.1 M Tris-HCl, pH 7.4, containing 50 mM KCl in the presence of either 1 mM NADPH or 1 mM NADH. Reactions were performed at 25 °C. Dicoumarol (30 μM) (*solid bar*) or superoxide dismutase (0.2 mg/ml) (*striped bar*) was added separately to test the specificity of the reactions. Values are expressed as the mean ± S.E. of three experiments.

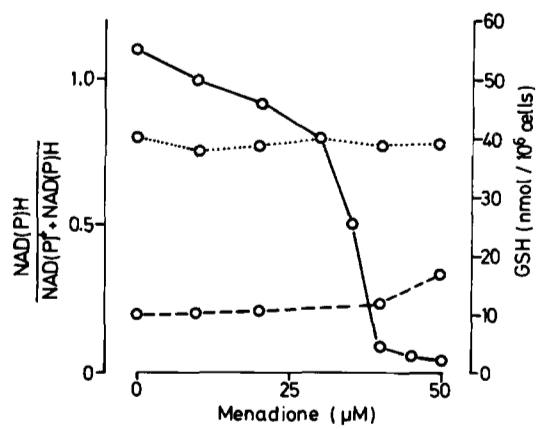

FIG. 4. **Low concentrations of menadione deplete GSH from isolated hepatocytes without affecting the redox state of pyridine nucleotides.** Hepatocytes were isolated from phenobarbital-treated rats and incubated with different concentrations of menadione. After 20 min, samples were taken and the GSH level (O——O), the NADPH/(NADP⁺ + NADPH) (O····O), and NADH/(NAD⁺ + NADH) (O– – –O) ratios determined. The results of one experiment typical of three are shown.

Up to this concentration the hepatocytes were relatively resistant to menadione-induced GSH loss, but in the 30–40 μM range a threshold value appears to have been reached. This could reflect saturation of the capacity of the hepatocytes to carry out two-electron reduction of menadione, since one-electron reduction resulting in the formation of $O_2^{\cdot-}$ and H_2O_2 is almost certainly responsible for the GSH loss (25). This result therefore suggests the preferential metabolism of menadione via the DT-diaphorase-mediated two-electron reduction pathway in isolated hepatocytes. Support for this idea also comes from a comparison of the apparent K_m values for menadione of cytosolic DT-diaphorase and microsomal NADPH-cytochrome P-450 reductase, which were determined to be 3.6 ± 1.0 and 43.0 ± 8.5 μM, respectively. (Values represent the mean ± S.E. of five separate determinations in isolated subcellular fractions.)

Potentiation of the Cytotoxic Effect of Menadione by Dicoumarol and Diethyldithiocarbamate—As shown in Fig. 5, depletion of cellular GSH during menadione metabolism was followed by rapid loss of cell viability measured by trypan blue uptake. The loss of both GSH and cell viability was more rapid and extensive with hepatocytes isolated from phenobarbital-treated rats than with those from either 3-MC-treated or untreated (control) animals. These findings support the hypothesis that one-electron reduction of menadione leading to redox cycling is responsible for the effects on both cellular GSH level and viability.

Further support for this hypothesis is presented in Fig. 6, which shows that both dicoumarol and diethyldithiocarbamate, an inhibitor of superoxide dismutase (26), potentiated menadione toxicity to isolated hepatocytes. This effect was most pronounced with dicoumarol, which caused a 3- to 5-fold increase in the toxicity of menadione at concentrations between 30 and 100 μM. The potentiation of menadione toxicity by diethyldithiocarbamate was less marked, but readily observable at higher menadione concentrations (Fig. 6). Neither dicoumarol (30 μM) not diethyldithiocarbamate (25 μM) had any effect on the viability of the hepatocytes in the absence of menadione (*cf.* zero time values in Fig. 6). The results confirm that both DT-diaphorase and superoxide dismutase protect isolated hepatocytes against the toxic effects of menadione.

Changes in Cellular Ca^{2+} Homeostasis during Menadione Metabolism—Early in the investigation we noticed that high concentrations (100–200 μM) of menadione caused the formation of a great number of small blebs on the surface of many of the hepatocytes. Formation of surface blebs occurred during the 1st h of the incubation and prior to any increase in trypan blue uptake or other indications of a decrease in cell viability.

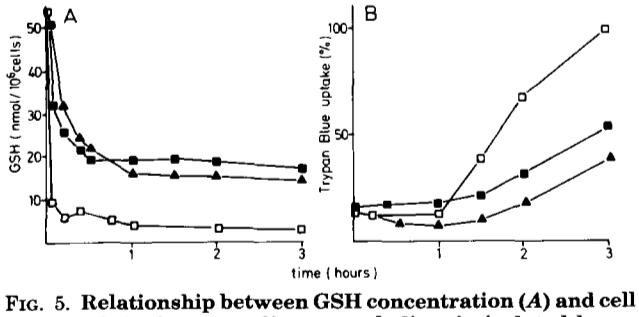

FIG. 5. **Relationship between GSH concentration (*A*) and cell viability (*B*) during menadione metabolism in isolated hepatocytes.** Hepatocytes isolated from control (▲), phenobarbital (□)- or 3-MC (■)-treated rats were incubated with menadione (50 μM) and dicoumarol (30 μM) for up to 3 h. At specific times the GSH concentration (*A*) and the percentage of cells taking up trypan blue (*B*) were determined. The values are expressed as the means of three separate experiments.

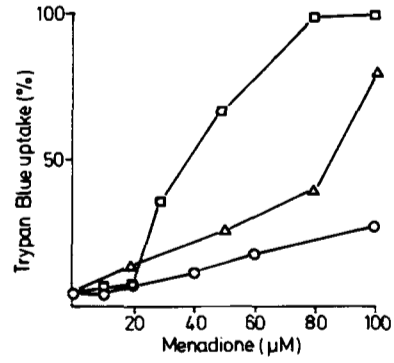

FIG. 6. **Potentiation of menadione cytotoxicity by dicoumarol and diethyldithiocarbamate.** Hepatocytes isolated from phenobarbital-treated rats were incubated (10^6 cells/ml) with varying concentrations of menadione alone (○), and with dicoumarol, 30 μM (□) or diethyldithiocarbamate, 25 μM (△) also present. After a 2-h incubation the viability of the hepatocytes was determined as the percentage of cells taking up trypan blue. The results of one experiment typical of three are shown.

This suggested to us that surface blebbing was a very early event in menadione cytotoxicity, which would be in agreement with our previous findings on the toxicity of bromobenzene in isolated hepatocytes (27). Moreover, we found that dicoumarol greatly potentiated the formation of surface blebs (Fig. 7*D*) at concentrations of menadione which caused only minor perturbations of hepatocyte surface structure (Fig. 7*B*). Dicoumarol (30 μM) alone had no effect on the surface morphology of the hepatocytes (Fig. 7*C*).

Similar alterations in the surface structure of isolated hepatocytes also occur during the metabolism of *t*-butylhydroperoxide, and we have recently reported (15) that these changes are associated with alterations in intracellular thiol and Ca^{2+} homeostasis. Fig. 8 shows the effects of menadione and dicoumarol, alone and in combination, on the levels of total Ca^{2+}, mitochondrial Ca^{2+} (A23187-releasable, following carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone-induced release of mitochondrial Ca^{2+}) in isolated hepatocytes. The addition of either menadione (50 μM) or dicoumarol (30 μM) led to a substantial decrease in total cell Ca^{2+}, but only the two in combination caused a complete loss (Fig. 8*A*). Dicoumarol caused a release of mitochondrial Ca^{2+} (Fig. 8*B*), but did not produce blebbing on the hepatocyte surface (Fig. 7*C*). Thus, the level of mitochondrial Ca^{2+} does not appear to directly control the surface structure of isolated hepatocytes. Both menadione and dicoumarol also caused an approximately 50% loss of extramitochondrial Ca^{2+} after 30–60 min of incubation, but the two together caused a total loss of extramitochondrial Ca^{2+} after only 20 min (Fig. 8*C*). This was associated with extensive blebbing of the hepatocytes which subsequently showed fragmentation and substantial loss of viability.

DISCUSSION

A schematic view of quinonoid drug metabolism in isolated hepatocytes is presented in Fig. 9. Although some quinones can be reduced directly by intracellular reductants, such as ascorbate and GSH, the majority are reduced enzymatically by flavoproteins in either a one-electron or two-electron transfer process (2, 3, 10). The one-electron reduction of quinones results in the formation of semiquinone radicals, which usually have a high affinity for O_2 and reduce it to $O_2^{\cdot-}$ (3, 9, 11). The quinone is regenerated in this process and a redox cycle is initiated forming large amounts of $O_2^{\cdot-}$ (9, 28, 29); $O_2^{\cdot-}$ subsequently dismutates to form H_2O_2 and O_2 in a reaction catalyzed by the superoxide dismutases (5–8, 30). However, $O_2^{\cdot-}$ and

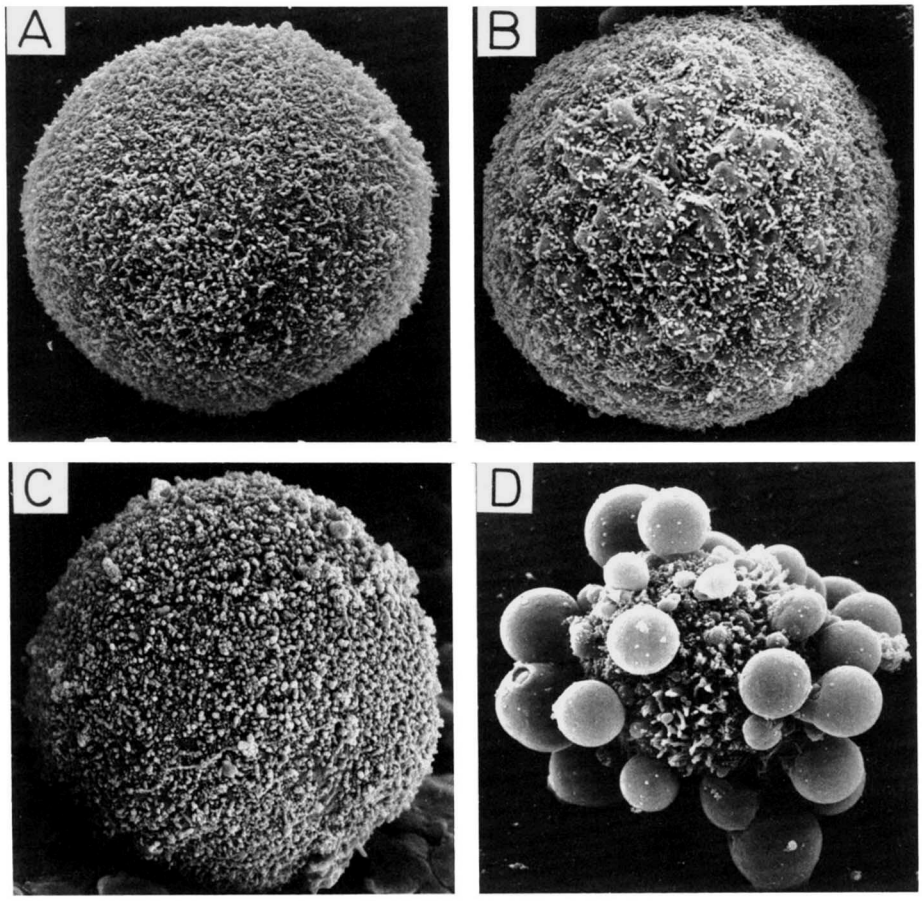

FIG. 7. **Effect of menadione and dicoumarol, alone and in combination, on the surface structure of isolated hepatocytes.** Hepatocytes from phenobarbital-treated rats were incubated (10^6 cells/ml) with (*A*) no addition; (*B*) menadione, 50 μM; (*C*) dicoumarol, 30 μM; and (*D*) menadione, 50 μM + dicoumarol, 30 μM for 20 min, and samples were processed for scanning electron microscopy. Typical hepatocytes are shown. Total magnifications: *A*, *B*, and *C* × 4000; *D* × 2500.

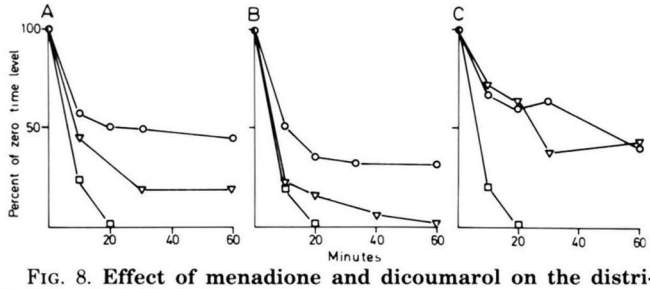

FIG. 8. **Effect of menadione and dicoumarol on the distribution of Ca^{2+} in isolated hepatocytes.** Hepatocytes from untreated rats were incubated (6 × 10^6 cells/ml) with menadione (○) and dicoumarol (▽), alone and in combination (□), at concentrations of 50 and 30 μM, respectively. The amounts of total (*A*), mitochondrial (*B*), and extramitochondrial (*C*) Ca^{2+} were then determined at specific time points using the technique described under "Experimental Procedures." The results of one experiment typical of three are shown.

H_2O_2 may also take part in metal-catalyzed reactions to form more toxic species of active oxygen, such as hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1\Delta g$ O_2) (8, 9, 28). Preliminary evidence suggests that $^1\Delta g$ O_2 is formed during the redox cycling of menadione in isolated hepatocytes,[2] and it is therefore likely that $OH^{\cdot}$ is also formed during this process. Both $O_2^{\cdot-}$ and more active forms of oxygen are potentially very toxic and are known to cause DNA strand breaks (31, 32), enzyme inhibition (33), lipid peroxidation (34, 35), and oxidation of thiol groups in proteins (36). However, lipid peroxidation is unlikely to be involved in the toxic effects of menadione, since this agent is a potent inhibitor of the propagation reactions of lipid peroxidation (37). Menadione did, however, cause the rapid loss of GSH during redox cycling in isolated hepatocytes (Ref. 25, see also "Results"), and it appears likely that the oxidation of GSH and important protein thiol groups is largely responsible for its cytotoxic effects.

In the present study we have shown that NADPH-cytochrome P-450 reductase is a more efficient catalyst of the one-electron reduction of menadione to menasemiquinone than NADH-cytochrome b_5 reductase. However, it is likely that the mitochondrial flavoprotein, NADH-ubiquinone oxidoreductase, may play a more important role for the redox cycling of other quinones, as recently suggested by Powis and coworkers (29). The contribution of each flavoprotein will largely be controlled by the one-electron reduction potential of the quinone (11, 29).

The two-electron reduction of menadione is catalyzed most effectively by the flavoprotein NAD(P)H: (quinone-acceptor) oxidoreductase, otherwise known as DT-diaphorase (2, 10). The results of the present study confirm the findings reported in Refs. 2 and 10 that DT-diaphorase reduces quinones to hydroquinones (diols) without the formation of semiquinone radical intermediates and $O_2^{\cdot-}$ (Fig. 9). Moreover, the affinity of menadione for hepatic DT-diaphorase was found to be more than 10 times greater than its affinity for microsomal NADPH-cytochrome P-450 reductase. DT-diaphorase may therefore protect against menadione cytotoxicity by competing with the potentially toxic one-electron reduction pathway.[3] Measurement of antimycin A-independent oxygen consumption by isolated hepatocytes allowed us to demonstrate that addition of dicoumarol, a potent inhibitor of DT-diaphorase

[2] H. Sies, personal communication.

[3] Since this report was submitted for publication a paper has been published (Lind, C., Hochstein, P., and Ernster, L. (1982) *Arch. Biochem. Biophys.* **216,** 178–185) in which data obtained with isolated subcellular fractions and purified enzymes are presented. Their results are consistent with ours in that they support the idea of DT-diaphorase acting as a cellular control device against semiquinone and superoxide radical formation and hence quinone toxicity.

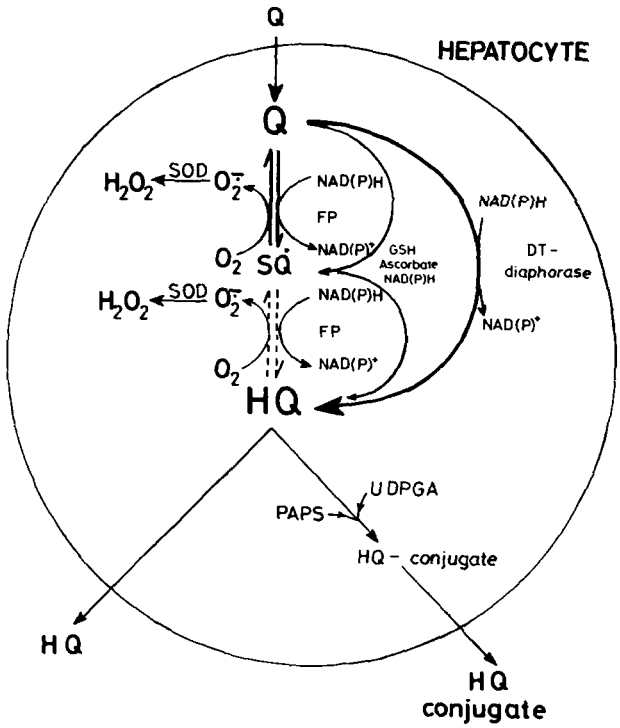

FIG. 9. **A schematic representation of quinonoid drug metabolism in isolated hepatocytes.** *Q*, quinone; *SQ*, semiquinone radical; *HQ*, hydroquinone (diol); *FP*, flavoprotein; *SOD*, superoxide dismutase; *PAPS*, 3′-phosphoadenosine-5′-phosphosulfate; *UDPGA*, uridine-5′-diphosphoglucuronic acid.

(10), greatly increased the availability of menadione for one-electron reduction. The results obtained by measurement of O_2^- production during menadione metabolism in isolated hepatocytes were, however, slightly more complex.

Derivatized forms of cytochrome *c* are presently the indicators of choice in measuring O_2^- formation in biological sytems (16, 38). However, one major drawback to their use is that they can also be reduced by other compounds, such as menadiol (10, 14). This limitation is clearly shown in the present study where we introduced extracellular acetylated cytochrome *c* to measure O_2^- release by isolated hepatocytes. Incubation of hepatocytes with menadione greatly stimulated the rate of reduction of acetylated cytochrome *c*, a large proportion of which was superoxide dismutase-inhibitable. This stimulatory effect of menadione on hepatocyte O_2^- formation is in sharp contrast to its inhibitory effect on O_2^- production by stimulated human neutrophils (39). The O_2^--generating ability of neutrophils is, however, dependent upon the NAD(P)H oxidoreductase complex in the plasma membrane, which appears to have an intrinsic quinone reductase activity (40). Moreover, the quinonoid compound dichlorophenol-indophenol competitively inhibits this O_2^--generating oxidoreductase (40). To the best of our knowledge no such oxidoreductase complex is present to any significant extent in the plasma membrane of rat hepatocytes. The hepatocyte and neutrophil systems are therefore obviously different and care should be taken when comparing the effects of menadione on different cell types.

From the results of the oxygen uptake measurements one would have expected dicoumarol to increase the production and release of O_2^- by hepatocytes in the presence of menadione. This increased production should have resulted in a higher rate of extracellular cytochrome *c* reduction which was counterbalanced to some extent by dicoumarol preventing the formation and release of menadiol. Careful analysis of the data presented in Fig. 3 shows that dicoumarol inhibited the release of menadiol from hepatocytes but had less than the expected stimulatory effect on O_2^- release. This apparently anomalous result can probably be explained by dicoumarol having some effect on the permeability of the hepatocyte plasma membrane to O_2^-. It has been calculated that only about 3% of the O_2^- formed during menadione metabolism by hepatocytes will be released (29). Thus, only a small nonspecific effect of dicoumarol on O_2^- permeability could be very significant. The development of specific indicators for O_2^- production in intact cells is probably required to clarify this question.

The addition of dicoumarol did, however, greatly potentiate the cytotoxicity of low concentrations of menadione, further indicating that DT-diaphorase plays a protective role in preventing the potentially toxic redox cycling of menadione in isolated hepatocytes. The idea that DT-diaphorase will compete with the single electron reduction pathway for menadione is also supported by our previous *in vitro* studies (9). However, the importance of this protective role of DT-diaphorase will depend upon the relative affinity of the quinonoid drug for DT-diaphorase and the flavoproteins catalyzing its one-electron reduction. For example, the quinonoid antitumor drug Adriamycin (doxorubicin) has a similar affinity for both DT-diaphorase and NADPH-cytochrome P-450 reductase *in vitro*,[4] and it is therefore unlikely that DT-diaphorase plays any major role in preventing the redox cycling of this drug.

Finally, dicoumarol not only potentiated the overall cytotoxicity of menadione, as determined by trypan blue exclusion, but also markedly abrogated its early toxic effects, as indicated by alterations in the surface structure of the hepatocytes. This potentiation appeared to be far greater than the blocking action of dicoumarol on DT-diaphorase could possibly cause alone. The studies on changes in intracellular Ca^{2+} distribution during menadione metabolism reported here would also tend to suggest that dicoumarol further potentiates menadione cytotoxicity by its mild uncoupling effect. Recent studies in our laboratory (15, 41) have shown that interference with mitochondrial Ca^{2+} homeostasis is also necessary if one is to observe a rapid onset of extensive surface blebbing following GSH depletion in isolated hepatocytes. Dicoumarol could therefore be potentiating the cytotoxicity of menadione in two ways: (*a*) by inhibiting its two-electron reduction to menadiol, thereby making more of the quinone available for potentially toxic redox cycling; and (*b*) by decreasing the ability of the mitochondria to control overall intracellular Ca^{2+} homeostasis. The importance of changes in intracellular Ca^{2+} homeostasis during quinonoid drug metabolism in isolated hepatocytes and other cell types is worthy of further investigation.

Acknowledgment—The authors are grateful to Sten Thorold for preparing the scanning electron micrographs.

REFERENCES

1. Driscoll, J. S., Hazard, G. F., Wood, H. B., and Goldin, A. (1974) *Cancer Chemother. Rep. Part 2* **4**, 1–362
2. Iyanagi, T., and Yamazaki, I. (1970) *Biochim. Biophys. Acta* **216**, 282–294
3. Bachur, N., Gordon, S. L., and Gee, M. V. (1978) *Cancer Res.* **38**, 1745–1750
4. Patel, K. B., and Willson, R. L. (1973) *J. Chem. Soc. Faraday Trans.* **69**, 814–825
5. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
6. Beauchamp, C., and Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4641–4646
7. Kellogg, E. W., III, and Fridovich, I. (1975) *J. Biol. Chem.* **250**, 8812–8817
8. Hassan, H. M., and Fridovich, I. (1979) *Arch. Biochem. Biophys.*

[4] H. Thor, M. T. Smith, P. Hartzell, and S. Orrenius, unpublished observations.

196, 385–395
9. Smith, M. T., Thor, H., and Orrenius, S. (1982) in *Microsomes, Drug Oxidation and Drug Toxicity* (Sato, R., and Kato, R., eds) Japan Scientific Societies Press, Tokyo, 605–612
10. Ernster, L. (1967) *Methods Enzymol.* **10,** 309–317
11. Powis, G., and Appel, P. L. (1980) *Biochem. Pharmacol.* **29,** 2567–2572
12. Ernster, L., Lind, C., Nordenbrand, K., Thor, H., and Orrenius, S. (1982) in *Oxygenases and Oxygen Metabolism* (Nozaki, M., Yamamoto, P., Ishimura, Y., Coon, M. J., Ernster, L., and Estabrook, R. W., eds) Academic Press, Tokyo, in press
13. Yasukochi, U., and Masters, B. S. S. (1976) *J. Biol. Chem.* **251,** 5337–5344
14. Lind, C., and Ernster, L. (1974) *Biochem. Biophys. Res. Commun.* **56,** 392–400
15. Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) *Proc. Natl. Acad. Sci. U. S. A.*, in press
16. Azzi, A., Montecucco, C., and Richter, C. (1975) *Biochem. Biophys. Res. Commun.* **65,** 597–607
17. Moldéus, P., Högberg, J., and Orrenius, S. (1978) *Methods Enzymol.* **52,** 60–71
18. Ernster, L., Siekevitz, P., and Palade, G. E. (1962) *J. Cell Biol.* **15,** 541–562
19. Thayer, W. S. (1977) *Chem. Biol. Interact.* **19,** 265–275
20. Saville, B. (1958) *Analyst* **83,** 670–672
21. Klingenberg, M. (1970) in *Methoden der Enzymatischen Analyze* (Bergmeyer, H. V., ed) p. 1975, Verlag-Chemie, Weinheim
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
23. Murphy, E., Coll, K., Rich, T. L., and Williamson, J. R. (1980) *J. Biol. Chem.* **255,** 6600–6608
24. Kendrick, N. C., Ratzlaff, R. W., and Blaustein, M. P. (1977) *Anal. Biochem.* **83,** 433–450
25. Eklöw, L., Thor, H., and Orrenius, S. (1981) *FEBS Lett.* **127,** 125–128
26. Heikkila, R. E., Cabbat, F. S., and Cohen, G. (1976) *J. Biol. Chem.* **251,** 2182–2185
27. Thor, H., and Orrenius, S. (1980) *Arch. Toxicol.* **44,** 31–43
28. Kappus, H., and Sies, H. (1981) *Experientia (Basel)* **37,** 1233–1241
29. Powis, G., Svingen, B. A., and Appel, P. (1981) *Mol. Pharmacol.* **20,** 387–394
30. Fridovich, I. (1975) *Annu. Rev. Biochem.* **44,** 147–159
31. Lesks, S. A., Lorentzen, R. J., and Ts'o, P. O. P. (1980) *Biochemistry* **19,** 3023–3028
32. Brawn, K., and Fridovich, I. (1981) *Arch. Biochem. Biophys.* **206,** 414–419
33. Searle, A. J., and Willson, R. L. (1980) *Int. J. Radiat. Biol.* **37,** 213–217
34. Bus, J. S., and Gibson, J. E. (1979) in *Reviews in Biochemical Toxicology* (Hodgson, E., Bend, J. R., and Philpot, R. M., eds) Vol. 1, pp. 125–149, Elsevier, Amsterdam
35. Tien, M., Svingen, B. A., and Aust, S. D. (1981) *Fed. Proc.* **40,** 179–182
36. McCord, J. M. (1979) in *Reviews in Biochemical Toxicology* (Hodgson, E., Bend, J. R., and Philpot, R. M., eds) Vol. 1, pp. 109–124, Elsevier, Amsterdam
37. Wills, E. D. (1972) *Biochem. Pharmacol.* **21,** 1879–1883
38. McCord, J. M., Crapo, J. D., and Fridovich, I. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M., and Fridovich, I., eds) pp. 11–18, Academic Press, New York
39. Gallin, J. I., Seligmann, B. E., Cramer, E. B., Schiffmann, E., and Fletcher, M. P. (1982) *J. Immunol.* **128,** 1399–1408
40. Green, T. R., and Schaefer, R. E. (1981) *Biochemistry* **20,** 7483–7487
41. Jewell, S. A., Bellomo, G., Thor, H., Orrenius, S., and Smith, M. T. (1982) *Science (Wash. D. C.)*, in press