Biochemical Pharmacology, Vol. 38, No. 16, pp. 2631–2637, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INTERCONVERSION OF NAD(H) TO NADP(H)

A CELLULAR RESPONSE TO QUINONE-INDUCED OXIDATIVE STRESS IN ISOLATED HEPATOCYTES

COLIN R. STUBBERFIELD and GERALD M. COHEN

Toxicology Unit, Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London, WC1N 1AX, U.K.

(*Received* 24 *November* 1988; *accepted* 24 *February* 1989)

Abstract—Quinones may be toxic by a number of mechanisms, including oxidative stress caused by redox cycling and arylation. This study has compared the cytotoxicity of four quinones, with differing abilities to arylate cellular nucleophiles and redox cycle, in relation to their effects on cellular pyridine nucleotides and ATP levels in rat hepatocytes. Non-toxic concentrations (50 μM) of menadione (redox cycles and arylates), 2-hydroxy-1,4-naphthoquinone (neither arylates nor redox cycles via a one electron reduction) and 2,3-dimethoxy-1,4-naphthoquinone (a pure redox cycler) all caused markedly similar changes in cellular pyridine nucleotides. An initial decrease in NAD^+ was accompanied by a small, transient increase in $NADP^+$ and followed by a larger, prolonged increased in NADPH and total $NADP^+$ + NADPH. At toxic concentrations (200 μM), the quinones caused an extensive depletion of NAD(H), an increase in levels of $NADP^+$ and an initial rise in total $NADP^+$ + NADPH, prior to a decrease in ATP levels and cell death. Nucleotide changes were not observed with non-toxic (20 μM) or toxic (100 μM) concentrations of *p*-benzoquinone (a pure arylator) and ATP loss accompanied or followed cell death. A novel mechanism for the activation of 2-hydroxy-1,4-naphthoquinone has been implicated. Our findings also suggest that a primary event in the response of the cell to redox cycling quinones is to bring about an interconversion of pyridine nucleotides, possibly mediated by an NAD^+ reduction, in an attempt to combat the effects of oxidative stress.

The mechanism of cytotoxicity of quinones has been extensively studied [1–3] and although many features of the mechanism have been described, the key determinants in the process remain to be elucidated. Quinones may exert their effects by a number of mechanisms including inhibition of mitochondrial electron transport [4], quinone-mediated electron transfer [5] and direct reaction with cellular macromolecules, particularly nucleophilic Michael addition to thiol and amino groups [6–8]. Most contemporary research, however, has emphasized the role of oxidative stress and redox cycling in the cytotoxicity [2, 9–11]. Quinones may be enzymically reduced by both one (microsomal NADPH–cytochrome P-450 reductase, microsomal NADH–cytochrome b_5 reductase and mitochondrial NADH–ubiquinone oxidoreductase) and two (DT-diaphorase) electron reductions, resulting in formation of their corresponding semiquinones or hydroquinones, respectively [2, 12, 13]. The semiquinone may be toxic *per se* or depending on its one electron redox potential, may react with molecular oxygen forming superoxide anion radical ($O_2^{\cdot-}$) and regenerating the parent quinone, which is then available to be rereduced and hence undergo a futile redox cycle [2, 3, 9–11]. The net result of this redox cycling is an oxidative stress resulting from disproportionate consumption of cellular reducing equivalents and the generation of active oxygen species ($O_2^{\cdot-}$, H_2O_2 and OH·). In contrast to the one electron reduction pathway, two electron reduction of quinones catalysed by DT-diaphorase is considered to be a detoxication pathway since the resulting hydroquinone may be conjugated and excreted [10, 14].

The loss of reduced pyridine nucleotides, through one and two electron reductases, is further aggravated by the removal of H_2O_2 through the GSH peroxidase/GSSG reductase cycle. Some of the oxidation is compensated for, however, by an activation of the hexose monophosphate shunt, increasing the supply of NADPH [2].

Exposure of hepatocytes to menadione (2-methyl-1,4-naphthoquinone) results in a rapid oxidation of intracellular glutathione (GSH), with the appearance of increased levels of GSSG and protein mixed disulphides [10, 15, 16]. This is accompanied by plasma membrane blebbing, a sustained rise in cytosolic free Ca^{2+} levels, and marked oxidation of NADPH [10, 17]. Based on our findings that menadione, H_2O_2 and dimethyl sulphate caused a depletion of intracellular NAD^+ which preceded a depletion of ATP and cytotoxicity, we have suggested that maintenance of intracellular NAD^+ may be a key factor in preserving cellular integrity [18].

A full understanding of the mechanism of toxicity of menadione is complicated because it may cause both oxidative stress via redox cycling and/or arylation of protein thiols [6, 7, 10, 19, 20]. In an attempt to identify which properties of menadione are responsible for its observed actions, we have compared its toxicity and its effects on nucleotide levels with those of three other quinones, i.e. *p*-benzoquinone (a pure arylator) [21], 2,3-dimethoxy-1,4-naphthoquinone (a pure redox cycler) [20] and 2-hydroxy-1,4-naphthoquinone, which neither arylates nor redox cycles following a one electron reduction

[22]. Of the three other quinones examined here, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone both behaved similarly to menadione causing an early depletion of NAD(H), which preceded loss of ATP and cell death, while the depletion following a toxic concentration of *p*-benzoquinone accompanied cell death with no effects on the nucleotide levels at non-toxic concentrations. Analysis of NADPH showed a rapid oxidation with menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, which was not observed with the arylating *p*-benzoquinone, and an increase in total $NADP^+$ + NADPH associated with the NAD(H) loss. These results strongly suggest an interconversion between the pyridine nucleotides.

MATERIALS AND METHODS

Collagenase, HEPES, firefly lantern extract, thiazolyl blue, phenazine ethosulphate, alcohol dehydrogenase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase and all nucleotides were obtained from Sigma Chemical Co. (Poole, U.K.). 2-Methyl-1,4-naphthoquinone, *p*-benzoquinone, 2-hydroxy-1,4-naphthoquinone and tributylamine were from Aldrich Chemical Co. (Gillingham, U.K.). 2,3-Dimethoxy-1,4-naphthoquinone was synthesized as previously described [20]. The remaining reagents were purchased from BDH Ltd (Poole, U.K.).

Cell isolation and incubation. Hepatocytes were prepared from male ICI Alderley Park strain Wistar rats (ICI plc, Alderley Park, U.K.) using Sigma type IV collagenase and incubated in rotating round bottomed flasks in a Krebs–Henseleit salt solution containing 12.5 mM HEPES buffer [23]. Cell viability was determined by Trypan blue exclusion in a 0.2% solution and samples were prepared and stored as previously described [18]. Protein was measured by the method of Lowry *et al.* [24].

Nucleotide determinations. Oxidized nucleotides were determined using spectrophotometric thiazolyl blue/phenazine ethosulphate-linked recycling assays [18, 25]. The NAD^+ assay contained ethanol (120 mM) and alcohol dehydrogenase (12.5 units/ml) in 0.1 M potassium phosphate buffer pH 7.6 and the $NADP^+$ assay glucose-6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (1 unit/ml) in 0.1 M glycylglycine/0.1 M nicotinamide/12.5 mM EDTA pH 7.4. Both assays contained thiazolyl blue and phenazine ethosulphate at concentrations of 50 and 200 μM, respectively. The nucleotide content of the sample was proportional to the rate of increase in absorbance at 570 nm.

NADH and NADPH were separated on a C18 reverse-phase column using isocratic elution with 0.2 M ammonium phosphate pH 6.0/17.87% methanol/0.13% tributylamine [26]. The eluent was monitored at 340 nm and the peak area calculated by integration.

ATP measurement. ATP levels were determined using a luciferase-linked luminescence method as described previously [18].

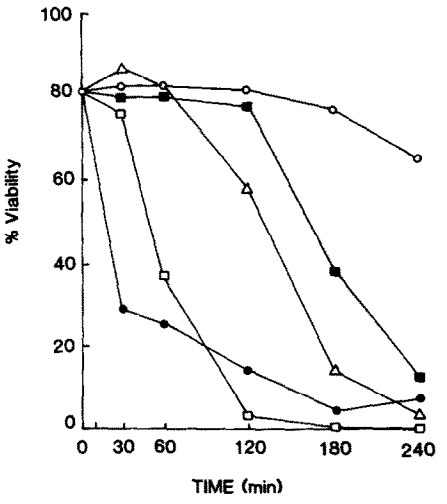

Fig. 1. Effects of quinones on cell viability. Hepatocytes were incubated either alone (○—○) or with menadione (200 μM; □—□), *p*-benzoquinone (100 μM; ●—●), 2,3-dimethoxy-1,4-naphthoquinone (200 μM; ■—■) or 2-hydroxy-1,4-naphthoquinone (200 μM; △—△). Viability was assessed as the percentage of cells excluding Trypan blue in a 0.2% solution. The results represent the mean of three separate preparations.

RESULTS

Effects of quinones on toxicity and pyridine nucleotides

All four quinones produced a concentration- and time-dependent cytotoxicity to freshly isolated hepatocytes (Fig. 1). The order of toxicity was benzoquinone > menadione > 2-hydroxy-1,4-naphthoquinone > 2,3-dimethoxy-1,4-naphthoquinone. Toxic concentrations caused a rapid depletion of NAD^+ and NADH (Fig. 2), which preceded cell death with menadione (200 μM), 2,3-dimethoxy-1,4-naphthoquinone (200 μM) and 2-hydroxy-1,4-naphthoquinone (200 μM), but appeared to accompany the loss of viability with *p*-benzoquinone (100 μM). However, with *p*-benzoquinone a precipitous fall in viability was observed with only a small increase in concentration, i.e. no toxicity was observed with 80 μM whereas 100 μM caused a rapid cell death. This complicated the identification of changes prior to cell death.

A small decrease in the level of $NADP^+$ + NADPH was observed in control hepatocytes during the 4 hr incubation (Fig. 2A). Prior to cell death, cytotoxic concentrations (200 μM) of menadione, 2-hydroxy-1,4-naphthoquinone and 2,3-dimethoxy-1,4-naphthoquinone caused extensive oxidation of NADPH, as seen by a fall in NADPH accompanied by an increase in $NADP^+$ which was followed by a decrease in $NADP^+$ associated with cell death (Fig. 2B, D, E). This was most evident with the less toxic quinones, i.e. 2,3-dimethoxy- and 2-hydroxy-1,4-naphthoquinone (Fig. 2D, E) where the time of onset of cell death occurred later in the incubation (Fig. 1). In contrast to the results with the other quinones, benzoquinone caused only a small increase

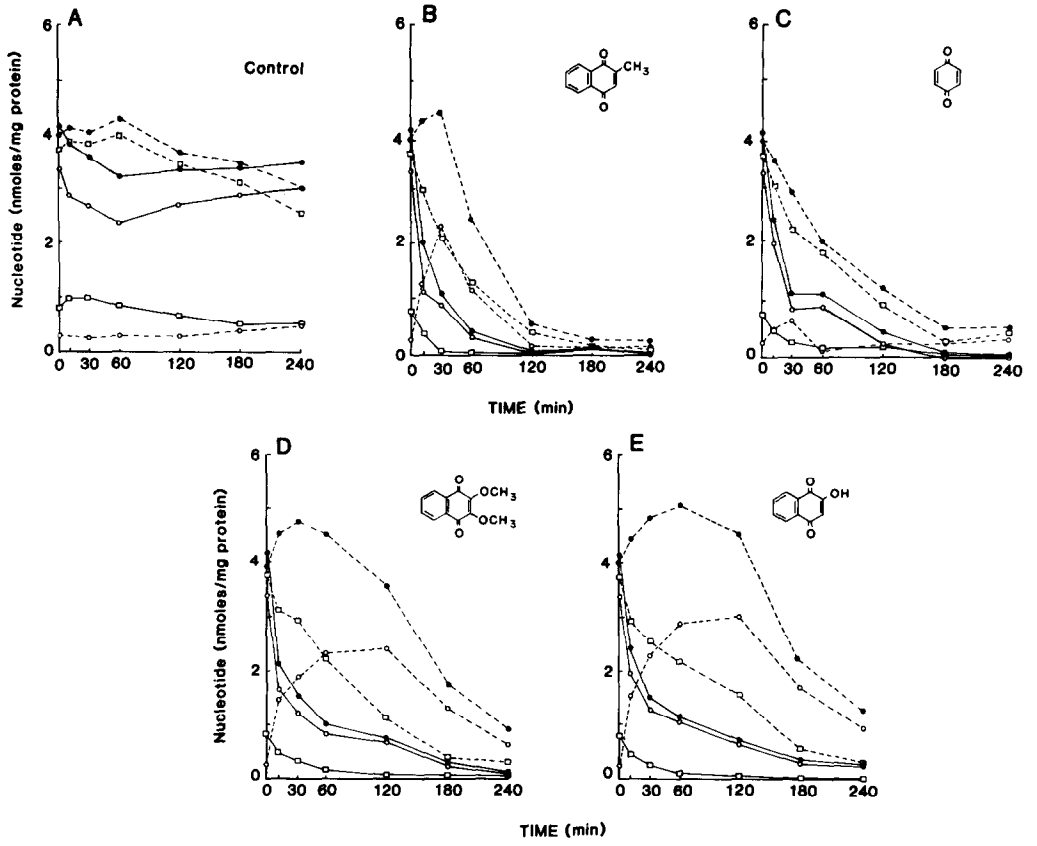

Fig. 2. Effects of toxic concentrations of quinones on oxidized and reduced cellular pyridine nucleotides. Hepatocytes were incubated either (A) alone or with (B) menadione (200 μM), (C) *p*-benzoquinone (100 μM), (D) 2,3-dimethoxy-1,4-naphthoquinone (200 μM) or (E) 2-hydroxy-1,4-naphthoquinone (200 μM). At the indicated times, intracellular NAD^+ (○—○), NADH (□—□), total NAD^+ + $NADH^+$ (●—●), $NADP^+$ (○- - -○), NADPH (□- - -□) and total $NADP^+$ + NADPH (●- - -●) were measured as described in Materials and Methods. The values represent the means from three separate hepatocyte preparations (average protein content = 2.16 mg/10^6 cells).

in $NADP^+$ but a large decrease in NADPH (Fig. 2C).

The increase in the levels of $NADP^+$ observed with cytotoxic concentrations of menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone were greater than the corresponding loss of NADPH, resulting in an apparent increase in total cellular $NADP^+$ + NADPH above control levels (Fig. 2B, D, E) which was subsequently lost with the onset of cell death. In order to further investigate this observation, nucleotide levels were analysed following exposure to non-toxic quinone concentrations (Fig. 3 and Table 1). At a non-toxic concentration of benzoquinone (20 μM) no effect on NAD(H) or NADP(H) was observed (Fig. 3B). However, non-toxic concentrations (50 μM) of menadione, 2-hydroxy- and 2,3-dimethoxy-1,4-naphthoquinone caused a prolonged increase in the levels of NADPH and total $NADP^+$ + NADPH accompanied by a small transient increase in $NADP^+$ over the first 10–30 min of incubation (Fig. 3A, C, D). The non-toxic concentrations had little or no effect on the levels of NADH but caused an initial rapid decline in the levels of NAD^+ which was slowly reversed during the course of the incubation (Fig. 3). This was seen most clearly with the cells exposed to 2,3-dimethoxy-1,4-naphthoquinone (Fig. 3C).

Effects of quinones on intracellular levels of ATP

Non-toxic concentrations of each quinone caused a small, short-lived decrease in the levels of ATP before recovering to control values by 30 min (data not shown). The reduction of the levels of ATP caused by toxic concentrations of menadione (200 μM), 2,3-dimethoxy-1,4-naphthoquinone (200 μM) and 2-hydroxy-1,4-naphthoquinone (200 μM) (Fig. 4) occurred after the observed changes in nucleotides (Fig. 2) but prior to cell death (Fig. 1), while the ATP loss caused by benzoquinone (100 μM) (Fig. 4) accompanied, or followed, cell death.

DISCUSSION

The changes in cellular pyridine nucleotide levels associated with exposure to menadione closely paralleled those produced by 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone.

Table 1. Effects of non-toxic concentrations of quinones on total pyridine nucleotide levels

	$NAD^+ + NADH$ (nmol/mg protein)	$NADP^+ + NADPH$ (nmol/mg protein)	Total pyridine nucleotides (nmol/mg protein)
Control	3.53 ± 0.23	4.05 ± 0.14	7.58 ± 0.36
Menadione (50 μM)	2.73 ± 0.41 (77)*	5.13 ± 0.36 (127)	7.87 ± 0.77 (104)
2,3-Dimethoxy-1,4-naphthoquinone (50 μM)	2.64 ± 0.17 (75)	5.55 ± 0.19 (137)	8.19 ± 0.06 (108)
2-Hydroxy-1,4-naphthoquinone (50 μM)	2.79 ± 0.32 (79)	4.71 ± 0.35 (116)	7.50 ± 0.41 (99)
p-Benzoquinone (20 μM)	3.50 ± 0.36 (99)	4.16 ± 0.24 (103)	7.66 ± 0.59 (101)

Nucleotide measurements were made after 30 min incubation in the presence of the quinones as described in Materials and Methods. The values are expressed as nmol/mg protein and represent mean ± SE from three separate hepatocyte preparations (average protein content = 2.03 mg/10^6 cells).
* The values in parentheses are the percentages of the corresponding controls.

Recently, we have reported a rapid loss of intracellular NAD^+ from hepatocytes following exposure to menadione, dimethyl sulphate and H_2O_2, which with the latter two compounds, but apparently not with menadione, was mediated by an activation of poly(ADP-ribose)polymerase [18]. In the present study, a similar fall in NAD^+ was observed in cells treated with 2,3-dimethoxy-1,4-naphthoquinone (Figs 2D and 3C) or 2-hydroxy-1,4-naphthoquinone (Figs 2E and 3D). As with menadione [18], preliminary studies with 3-aminobenzamide, an inhibitor of poly(ADP-ribose)polymerase, showed no effect on NAD^+ depletion or on the toxicity of these quinones (data not shown).

Previous studies have reported that low concentrations of menadione (10–50 μM) have no effect on the $NADPH/NADP^+ + NADPH$ ratio [10] and that higher, but still non-toxic concentrations (100 μM), cause an initial depletion of NADPH followed by an increase above controls [17]. In the present study, a non-toxic concentration of menadione (50 μM) caused a small, transient increase in $NADP^+$, followed by a more extensive and prolonged increase in NADPH and $NADP^+ + NADPH$ (Fig. 3A) whereas toxic concentrations (200 μM) caused a greater increase in $NADP^+$, but the increases in NADPH and $NADP^+ + NADPH$ were less marked because of the onset of cell death (Fig. 2B). Similar changes were observed with cells treated with 2,3-dimethoxy-1,4-naphthoquinone (Figs 2D and 3C) and 2-hydroxy-1,4-naphthoquinone (Figs 2E and 3D), while *p*-benzoquinone had little or no effect on any of the nucleotide levels prior to cell death (Figs 2C and 3B).

The possible fate of NAD(H) following menadione treatment has been discussed previously in relation to activation of glycohydrolases [18], and whilst the very rapid turnover of this nucleotide in hepatocytes [27] suggests the quinones may be decreasing its synthesis, careful consideration of our results strongly support another mechanism. With both non-toxic and toxic concentrations of menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, a striking coincidence in the fall of NAD^+ with a rise in total NADPH(H) was observed such that total pyridine nucleotide levels remained constant (Table 1). These results support the hypothesis that the initial NAD^+ depletion is due to an interconversion of pyridine nucleotides, with a synthesis of NADPH at the expense of NAD^+. The sequence of events may be envisaged as an initial conversion of NAD^+ to $NADP^+$ (10 min time points in Fig. 3A, C, D) followed by a reduction of $NADP^+$ to NADPH (30–60 min time points in Fig. 3A, C, D). Synthesis of $NADP^+$ from NAD^+ could occur by a number of indirect routes, but the simplest mechanism would be mediated by NAD^+ kinase. This enzyme is specific for the oxidized form of the nucleotide and has been found in a wide range of species and tissues including pigeon liver [28]. The mechanism by which the quinones may activate NAD^+ kinase is unclear but may involve calmodulin [29, 30]. Thus the rise in cytosolic free Ca^{2+} observed with the quinones, as well as other treatments which induce oxidative stress [31], also increases NAD^+ kinase activity through an activation of calmodulin. Kinase activity is also inhibited by NADPH [32], in a similar manner to the control of glucose-6-phosphate dehydrogenase activity. Thus the increase in NAD^+ kinase activity may parallel that of the hexose monophosphate shunt with a decrease in the $NADPH/NADP^+$ ratio associated with redox cycling [28, 33].

Reduction of $NADP^+$ may be effected by the activities of various enzymes including the hexose monophosphate shunt, isocitrate dehydrogenase, the malate shuttle or transhydrogenase reactions [34, 35]. It is well documented that an oxidative stress in cells from a number of different tissues including blood, liver, lung and brain results in an increase in the activity of the hexose monophosphate shunt, generally measured by an increase in the oxidation of [1-^{14}C]glucose [33, 36]. Such an increase has been demonstrated in hepatocytes exposed to menadione [17]. The interconversion of NAD^+ to $NADP^+$ described in this study, together with an increased rate of $NADP^+$ reduction, provides the cell with a rapid mechanism for increasing levels of NADPH, required for GSSG reduction and quinone metabolism in the defence of the cell.

The characteristic nucleotide changes, i.e. initial decrease in NAD^+ and a small transient increase in $NADP^+$ followed by a more prolonged and extensive

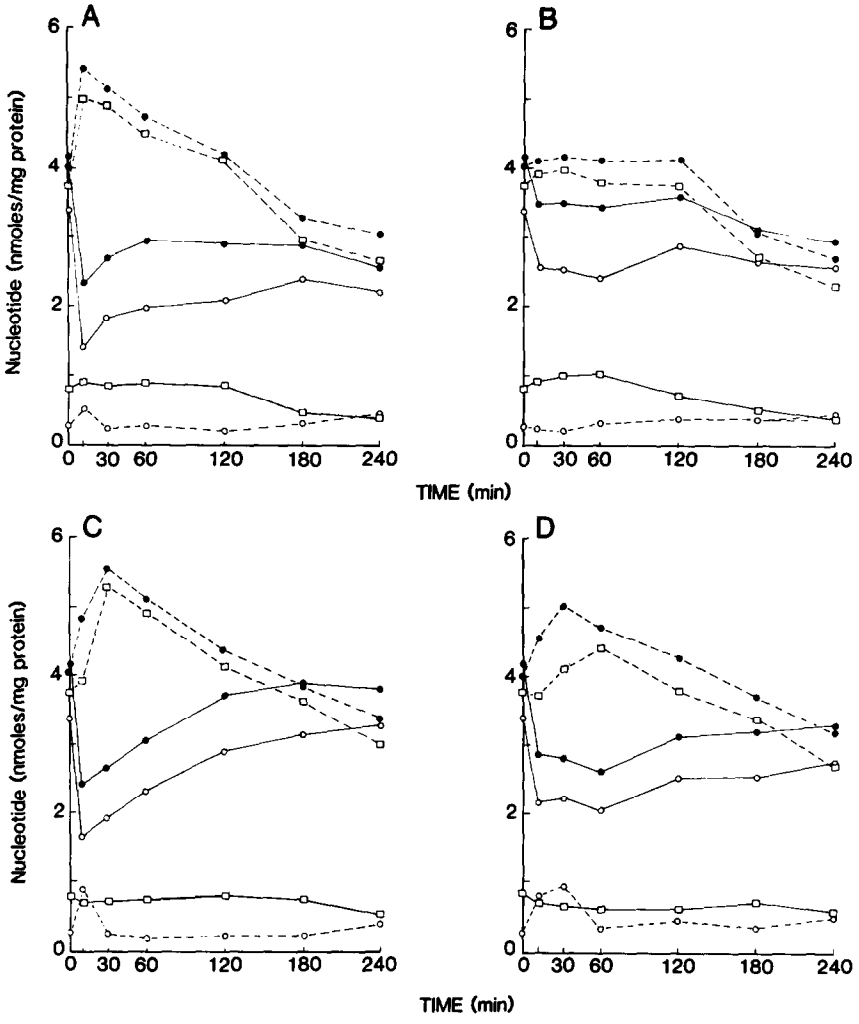

Fig. 3. Effects of non-toxic concentrations of quinones on cellular pyridine nucleotides. Hepatocytes were incubated with (A) menadione (50 μM), (B) *p*-benzoquinone (20 μM), (C) 2,3-dimethoxy-1,4-naphthoquinone (50 μM) or (D) 2-hydroxy-1,4-naphthoquinone (50 μM). Cellular nucleotides were measured as described in Materials and Methods. The symbols used are the same as those described in the legend to Fig. 2. The values represent the means of three separate hepatocyte preparations (average protein content = 2.03 mg/10^6 cells).

increase in NADPH, induced by non-toxic concentrations of 2,3-dimethoxy-1,4-naphthoquinone, menadione and 2-hydroxy-1,4-naphthoquinone (Fig. 3A, C, D) appear to be changes indicative of oxidative stress. These results would be expected from 2,3-dimethoxy-1,4-naphthoquinone, which has been shown to be a pure redox cycler, i.e. it is readily reduced by liver microsomes or intact hepatocytes to 2,3-dimethoxy-1,4-naphthosemiquinone anion free radical, which reacts with O_2 forming superoxide anion radical and regenerating the parent quinone, so establishing a futile redox cycle [20]. Menadione may both arylate nucleophiles [6, 7, 19, 20] and induce oxidative stress by redox cycling [10, 15, 16, 19, 20] and its effects on cellular nucleotides in the present study would suggest that many of its intracellular effects are due to redox cycling rather than arylation, in agreement with other studies [10, 15, 16, 37], although a critical role for arylation reactions in the overall cytotoxicity cannot be ruled out. The arylation of cellular nucleophiles by benzoquinone causes a rapid cell death (Fig. 1) without any alterations in cellular nucleotides prior to cell death.

Most interesting were the nucleotide changes induced by 2-hydroxy-1,4-naphthoquinone (Figs 2E and 3D) which, according to the results obtained with the other quinones, were indicative of oxidative stress. Our previous studies with this compound had shown it to cause a concentration-dependent depletion of intracellular GSH which preceded toxicity [. Its mechanism of toxicity seemed to be different from other quinones, however, as it did not appear to involve an initial one electron reduction to its corresponding semiquinone followed by $O_2^{\cdot-}$ production and redox cycling. This may be due to it being a very poor substrate for cellular one electron reductases such as NADPH–cytochome P-450

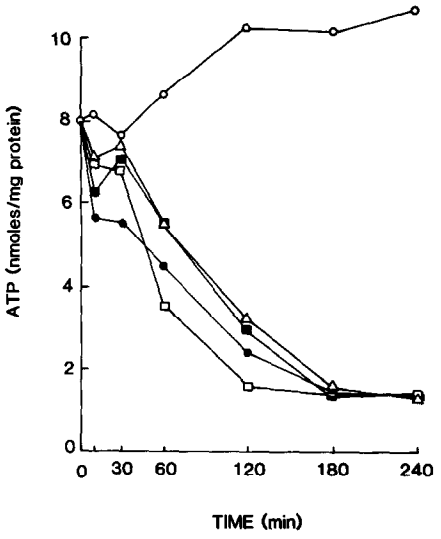

Fig. 4. Effects of toxic concentrations of quinones on cellular ATP levels. Hepatocytes were incubated either alone (○—○) or in the presence of menadione (200 μM; □—□), *p*-benzoquinone (100 μM; ●—●), 2,3-dimethoxy-1,4-naphthoquinone (200 μM; ■—■) or 2-hydroxy-1,4-naphthoquinone (200 μM; △—△). ATP was determined by a luciferase-linked chemiluminescence method. The values represent the mean of three separate preparations (average protein content = 2.16 mg/10^6 cells).

reductase, in part because of its very low one electron reduction potential (E_7^1-415 mV) [22]. Thus 2-hydroxy-1,4-naphthoquinone induces an oxidative stress in hepatocytes similar to that observed by other quinones, while its mechanism of activation appears to be different from many other quinones. Preliminary results suggest that it may be activated by a two electron reduction catalysed by DT-diaphorase to its hydroquinone, which then rapidly autooxidizes producing hydrogen peroxide and inducing an oxidative stress (Gant, Stubberfield and Cohen, manuscript in preparation).

In summary, exposure of hepatocytes to quinones including menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone causes characteristic changes in cellular pyridine nucleotides indicative of oxidative stress; these effects were not observed with *p*-benzoquinone, which acts by arylation. These changes, including an interconversion of pyridine nucleotides, possibly mediated by NAD^+ kinase, followed by a more prolonged elevation in NADPH, increase the cell's ability to cope with an oxidative stress. 2-Hydroxy-1,4-naphthoquinone induces an oxidative stress by a mechanism other than that commonly ascribed to other quinones.

Acknowledgements—We thank Mr S. Forrow and Dr E. Zaera for technical help. CRS is in receipt of a M.R.C. Studentship. We thank Mrs M. Fagg for the typing of the manuscript and Mr D. King for preparation of the figures.

REFERENCES

1. Webb JL (Ed.), In: *Enzyme and Metabolic Inhibitors*, Vol III, p. 445. Academic Press, New York, 1966.
2. Kappus H and Sies H, Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**: 1233–1241, 1981.
3. Smith MT, Evans CG, Thor H and Orrenius S, Quinone induced oxidative injury to cells and tissues. In: *Oxidative Stress* (Ed. Sies H), pp. 91–113. Academic Press, London, 1985.
4. Phelps OC and Crane FL, Inhibition of mitochondrial electron transport by hydroxy substituted 1,4-quinones. *Biochemistry* **14**: 116–122, 1975.
5. De Groot H, Noll T and Sies H, Oxygen dependence and subcellular partitioning of hepatic menadione-mediated oxygen uptake. Studies with isolated hepatocytes, mitochondria and microsomes from rat liver in an oxystat system. *Arch Biochem Biophys* **243**: 556–562, 1985.
6. Nickerson WJ, Falcone G and Strauss G, Studies on quinone thioethers I. Mechanism of formation and properties of thiodone. *Biochemistry* **2**: 537–543, 1963.
7. Ross D, Thor H, Orrenius S and Moldeus P, Interaction of menadione with glutathione. *Chem Biol Interact* **55**: 177–184, 1985.
8. Finley KT, The addition and substitution chemistry of quinones. In: *The Chemistry of the Quinoid Compounds* (Ed. Patai S), Part II, p. 878. John Wiley, London, 1974.
9. Sics H (Ed.), *Oxidative Stress*. Academic Press, London, 1985.
10. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. *J Biol Chem* **257**: 12419–12425, 1982.
11. Cohen GM and d'Arcy Doherty M, Free radical mediated cell toxicity by redox cycling chemicals. *Br J Cancer* **55**: 46–52, 1987.
12. Kappus H, Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. *Biochem Pharmacol* **35**: 1–7, 1986.
13. Powis G, Svingen BA and Appel P, Quinone stmulated superoxide formation by subcellular fractions, isolated hepatocytes and other cells. *Mol Pharmacol* **20**: 387–394, 1981.
14. Lind C, Hochstein P and Ernster L, DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch Biochem Biophys* **216**: 178–185, 1982.
15. Di Monte D, Ross D, Bellomo G, Eklow L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334–342, 1984.
16. Bellomo G, Mirabelli F, Di Monte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. *Biochem Pharmacol* **36**: 1313–1320, 1987.
17. Smith PF, Alberts DW and Rush GF, Menadione-induced oxidative stress in hepatocytes isolated from fed and fasted rats: the role of NADPH-regenerating pathways. *Toxicol Appl Pharmacol* **89**: 190–201, 1987.
18. Stubberfield CR and Cohen GM, NAD^+ depletion and cytotoxicity in isolated hepatocytes. *Biochem Pharmacol* **37**: 3967–3974, 1988.
19. Miller MG, Rodgers A and Cohen GM, Mechanisms of toxicity of naphthoquinones to isolated hepatocytes. *Biochem Pharmacol* **35**: 1177–1184, 1986.
20. Gant TW, Rao DNR, Mason RP and Cohen GM, Redox cycling and sulphydryl arylation; their relative importance in the mechanism of quinone cytotoxicty to isolated hepatocytes. *Chem Biol Interact* **65**: 157–173, 1988.
21. Rossi L, Moore GA, Orrenius S and O'Brien PJ, Quinone toxicity in hepatocytes without oxidative stress.

Arch Biochem Biophys **251**: 25–35, 1986.
22. d'Arcy Doherty M, Rodgers A and Cohen GM, Mechanisms of toxicity of 2- and 5-hydroxy-1,4-naphthoquinone; absence of a role for redox cycling in the toxicity of 2-hydroxy-1,4-naphthoquinone to isolated hepatocytes. *J Appl Toxicol* **7**: 123–129, 1987.
23. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52**: 60–71, 1978.
24. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
25. Bernofsky C and Swan M, An improved cycling assay for nicotinamide adenine dinucleotide. *Analyt Biochem* **53**: 452–458, 1973.
26. Kalhorn TF, Thummel KE, Nelson SD and Slattery JT, Analysis of oxidized and reduced pyridine dinucleotides in rat liver by high-performance liquid chromatography. *Analyt Biochem* **151**: 343–347, 1985.
27. Sies H (Ed.), Nicotinamide nucleotide compartmentation. In: *Metabolic Compartmentation*, pp. 205–231. Academic Press, London, 1982.
28. Kaplan NO, The role of pyridine nucleotides in regulating cellular metabolism. *Curr Top Cell Regul* **26**: 371–381, 1985.
29. Anderson JM and Cormier MJ, Calcium-dependent regulation of NAD kinase. *Biochem Biophys Res Commun* **84**: 595–602, 1978.
30. Epel G, Patton G, Wallace RW and Cheung WY, Calmodulin activates NAD kinase of sea urchin eggs: an early event of fertilization. *Cell* **23**: 543–549, 1981.
31. Di Monte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch Biochem Biophys* **235**: 343–350, 1984.
32. Oka H and Field JB, Inhibition of rat liver nicotinamide adenine dinculeotide kinase by reduced nicotinamide adenine dinculeotide phosphate. *J Biol Chem* **243**: 815–819, 1968.
33. Hoskin FCG and Von Eschen C, Stimulation by quinones of initial pentose phosphate pathway steps in soluble brain preparations. *Arch Biochem Biophys* **103**: 111–116, 1963.
34. Thurman RG and Kauffman FC, Factors regulating drug metabolism in intact hepatocytes. *Pharmacol Rev* **31**: 229–251, 1980.
35. Reed DJ, Regulation of reductive processes by glutathione. *Biochem Pharmacol* **35**: 7–13, 1986.
36. Rose MS, Smith LL and Wyatt I, The relevance of pentose phosphate pathway stimulation in rat lung to the mechanism of paraquat toxicity. *Biochem Pharmacol* **25**: 1763–1767, 1976.
37. Thor H, Mirabelli F, Salis A, Cohen GM, Bellomo G and Orrenius S, Alterations in hepatocyte cytoskeleton caused by redox cycling and alkylating quinones. *Arch Biochem Biophys* **266**: 397–407, 1988.