Biochemical Pharmacology, Vol. 33, No. 15, pp. 2367–2370, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

ENZYMATIC AND NON-ENZYMATIC REDUCTION OF *N*-ACETYL-*p*-BENZOQUINONE IMINE AND SOME PROPERTIES OF THE *N*-ACETYL-*p*-BENZOSEMIQUINONE IMINE RADICAL

GARTH POWIS,*† BRUCE A. SVINGEN,* DAVID C. DAHLIN‡ and SIDNEY D. NELSON‡
*Department of Oncology, Mayo Clinic, Rochester, MN 55905; and ‡Department of Medical Chemistry, University of Washington, Seattle, WA 98195, U.S.A.

(*Received* 6 *June* 1983; *accepted* 28 *November* 1983)

Abstract—*N*-Acetyl-*p*-benzoquinone imine (NAPQI) is the postulated hepatotoxic intermediate in acetaminophen overdosage. NAPQI was rapidly metabolized by NADPH–cytochrome P-450 reductase, with an apparent K_m of 1.8 to 4.0 μM and an apparent V_{max} of 29.4 μmoles per min per mg, and exhibited substrate inhibition of metabolism at NAPQI concentrations above 10 μM. NADPH was oxidized by NAPQI at a slower rate in the absence of enzyme. NAPQI did not appear to undergo redox cycling at an appreciable rate to form superoxide, and it did not stimulate oxygen utilization or superoxide release by rat isolated hepatocytes. Electron spin resonance studies failed to show formation of a free radical by chemical or enzymatic reduction of NAPQI under anaerobic conditions in aqueous media.

N-Acetyl-*p*-benzoquinone imine (NAPQI) (Fig. 1) has been proposed as the ultimate toxic metabolite in acetaminophen overdosage [1, 2]. Synthesis of pure NAPQI has been reported recently, and the quinone imine has been found to be considerably more toxic than acetaminophen to isolated hepatocytes and when administered i.p. to mice [3]. Formation of the *N*-acetyl-*p*-benzosemiquinone imine radical ($NAPQI^{\cdot}$) has been implicated in spontaneous decomposition of NAPQI [3]. Enzymatic one-electron reduction of NAPQI might also lead to formation of this radical and, under aerobic conditions, to the formation of oxygen radicals, as has been reported for simple quinones [4]. We report studies on the enzymatic reduction of NAPQI by purified flavoprotein and whole cells and describe the results of electron spin resonance spectroscopy studies.

MATERIALS AND METHODS

Enzyme and hepatocyte studies. NADPH–cytochrome P-450 (cytochrome *c*) reductase (EC 1.6.2.4) was prepared from the livers of phenobarbitone (80 mg/kg, i.p., for 3 days) -treated male Sprague–Dawley rats by the method of Yasukochi and Masters [5]. Specific activity of NADPH–cytochrome P-450 reductase was 67.9 units/mg protein. Incubation mixtures contained 0.1 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, NADPH–cytochrome P-450

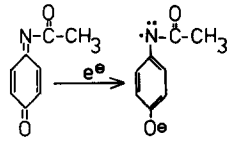

Fig. 1. NAPQI and its postulated reduction to $NAPQI^{\cdot}$.

reductase, 0.4 to 8 μg/ml, and, where appropriate, 1.2 to 6.0 μM cytochrome *c* at room temperature in a final volume of 3 ml. NAPQI in 10 μl dimethyl sulfoxide to give 0.5 μM to 0.1 mM or 2,5-dimethyl-*p*-benzoquinone in 10 μl dimethyl sulfoxide to give 0.1 mM was added immediately prior to initiating the reaction with 50 μM NADPH. Oxidation of NADPH was measured at 340 nm and reduction of cytochrome *c* at 550 nm. Products of non-enzymatic NAPQI reduction by NADPH were determined by reverse phase high performance liquid chromatography (HPLC) with an Altex ultrasphere ODS 5 μm column (4.6 mm + 25 cm). The solvent composition was 89% 0.075 M KH_2PO_4, 10% MeOH and 17% acetic acid. With a flow rate of 2 ml/min, acetaminophen eluted at 7.1 min, benzoquinone at 6.4 min, and NAPQI at 7.9 min. Superoxide formation was measured as reduction of 60 μM acetylated cytochrome *c* [6] at 550 nm at 37° in the presence and absence of superoxide dismutase, 66 μg/ml, as described previously [4]. Reaction was initiated in this case by addition of NADPH. Rat isolated hepatocytes were prepared by the method of Stewart and Inaba [7] and had a viability, measured by trypan blue exclusion, routinely more than 80%. Hepatocytes were incubated at 0.2 to 3×10^6 viable cells/ml in 3 ml of Dulbecco's phosphate-buffered saline containing 10 mM glucose. NAPQI or 2,5-dimethyl-*p*-benzoquinone in 10 μl dimethyl sulfoxide was added to give final concentrations of 0.1 mM. Oxygen utilization was measured using a Clark-type oxygen electrode (model 53, Yellow Springs Instruments, New York, NY), and superoxide formation was measured by reduction of 60 μM acetylated cytochrome *c* at 550 nm in the absence and presence of superoxide dismutase, 66 μg/ml, as previously described [8].

Electron spin resonance. Electron spin resonance (ESR) spectra were recorded on a Bruker ER-420

‡Author to whom reprint requests should be addressed.

ESR spectrometer using a quartz capillary sample cuvette (i.d. 0.2 cm). Instrument settings were: field set, 3410 G; microwave frequency, 9.375 GHz; modulation/receiver frequency, 100 kHz; microwave attenuation, 20 dB; and detector current, 200 μA. Incubations were conducted under anaerobic conditions at room temperature with a system containing 5 mM NAPQI in 0.15 M KCl, 10 mM potassium phosphate buffer, pH 7.0, NADPH-cytochrome P-450 reductase, 1.4 units/ml, and 1 mM NADPH. Alternatively, NAPQI was reduced with a 75% stoichiometric amount of $NaBH_4$. Chemical reduction was also conducted in dimethyl sulfoxide. The ESR spectrometer was calibrated using 1 mM sodium hydrosulfite or the 5′,5-dimethyl-1-pyrrole-*N*-oxide spin trapped adduct of hydroxyl radical generated by Fenton reagent [9].

Chemicals. NAPQI was synthesized as previously described [3]. 2,5-Dimethyl-*p*-benzoquinone was purchased from Eastman Kodak, Rochester, NY, and dimethyl sulfoxide from Burdick & Jackson, Muskegan, MI. NADPH, cytochrome *c* and bovine blood superoxide dismutase were obtained from the Sigma Chemical Co., St. Louis, MO.

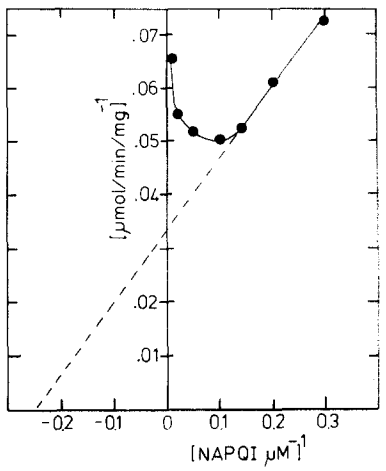

Fig. 2. Lineweaver–Burk plot for metabolism of NAPQI by NADPH–cytochrome P-450 reductase. Reaction conditions are described in the text. NADPH oxidation was measured at 340 nm at room temperature. Allowance was made for non-enzymatic oxidation of NADPH by NAPQI. NAPQI was added immediately before initiating the reaction with NADPH.

RESULTS

Enzymatic and non-enzymatic reduction. NADPH undergoes appreciable oxidation by NAPQI in the absence of enzyme. At an NAPQI concentration of 100 μM and an NADPH concentration of 5 μM the rate of NADPH oxidation at room temperature is 10.5 nmoles/min. The products of NAPQI reduction by NADPH measured by reverse phase HPLC were acetominophen 13%, benzoquinone 0.1% and, the remainder, a polymeric material, the majority of which was identified by mass spectrometry as a dimer (a future publication will deal with these studies in more detail). The kinetics of NAPQI metabolism by NADPH–cytochrome P-450 reductase are shown in Fig. 2. The apparent K_m was 4.0 μM and the apparent V_{max} 29.4 μmoles per min per mg. There was evidence of substrate inhibition of metabolism by NAPQI concentrations above about 10 μM. Because of the difficulty of accurately measuring K_m based on low NAPQI concentrations, an alternative approach was adopted of measuring the inhibition of NADPH–cytochrome P-450 reductase mediated cytochrome *c* reduction by low concentrations of NAPQI. Inhibition of cytochrome *c* reduction by NAPQI was time dependent. As NAPQI was itself metabolized by NADPH–cytochrome P-450 reductase, inhibition diminished and cytochrome *c* reduction approached, although never attained, the rate of reduction in the absence of NAPQI (Fig. 3). A Lineweaver–Burk plot of initial rates of cytochrome *c* reduction is shown in Fig. 4. Inhibition of cytochrome *c* reduction by NAPQI shows some characteristics of non-competitive inhibition with failure of all plots to intersect on the ordinate but, if competitive inhibition is assumed, a K_I of about 1.8 μM is obtained. Despite the relatively rapid rate of NAPQI metabolism by NADPH–cytochrome P-450 reductase, no superoxide formation could be detected as measured by reduction of acetylated cytochrome *c*. NAPQI blocked superoxide formation by 2,5-dimethylbenzoquinone, a good substrate for reduction by NADPH–cytochrome P-450 reductase [4] (Table 1).

Hepatocyte oxygen utilization and superoxide release. Using isolated rat hepatocytes, NAPQI had no effect upon oxygen utilization but completely blocked the increase in oxygen utilization produced by 2,5-dimethylbenzoquinone. NAPQI did not stimulate superoxide release by isolated hepatocytes and completely blocked the increase in superoxide release produced by 2.5-dimethylbenzoquinone. Typical traces are shown in Fig. 5, and mean values from several experiments are shown in Table 2. Acetaminophen did not stimulate release of superoxide by isolated hepatocytes and did not block superoxide release produced by 2,5-dimethylbenzoquinone (results not shown).

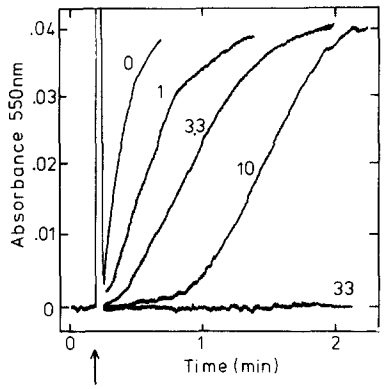

Fig. 3. Time dependence of inhibition of cytochrome *c* reduction by NADPH-cytochrome P-450 reductase by NAPQI. Incubation conditions are described in the text. NADPH, 0.05 mM, was added at the arrow to start the reaction. Cytochrome *c* reduction was followed at 550 nm and room temperature. Values next to recordings are NAPQI concentrations in μM.

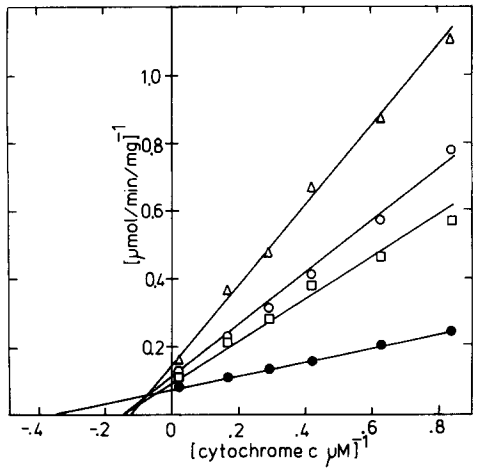

Fig. 4. Lineweaver–Burk plot of inhibition of cytochrome *c* reduction by NADPH–cytochrome P-450 reductase by NAPQI. Incubation mixtures are described in the text. The reaction was followed as the reduction of cytochrome *c* at 550 nm at room temperature: (●) no NAPQI; (□) 0.5 μM NAPQI; (○) 1 μM NAPQI, and (△) 3.3 μM NAPQI. NAPQI was added immediately before initiating the reaction with NADPH.

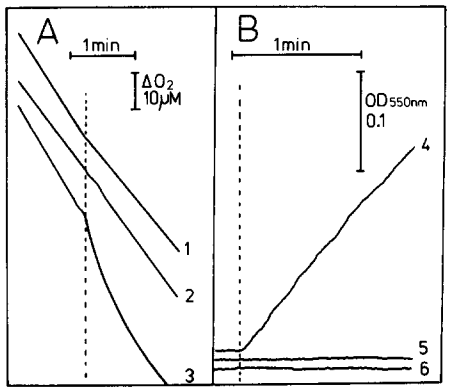

Fig. 5. Inhibition by NAPQI of rat isolated hepatocyte quinone-stimulated O_2 utilization and superoxide release. (A) Oxygen utilization. Incubation contained 3×10^6 viable hepatocytes/ml in phosphate-buffered saline, 10 mM glucose at 37°. Key: (1) control hepatocytes, 10^{-4} M NAPQI added at dotted line; (2) hepatocytes with 10^{-4} NAPQI, 10^{-4} M 2,5-dimethylbenzoquinone added at dotted line; and (3) control hepatocytes, 2,5-dimethylbenzoquinone added at dotted line. (B) Superoxide release measured as reduction of acetylated cytochrome *c* at 550 nm. Incubation contained 0.2×10^6 cells/ml in phosphate-buffered saline, 10 mM glucose at 37°. Key: (4) control hepatocytes, 10^{-4} M 2,5-dimethylbenzoquinone added at dotted line; (5) hepatocytes with 66 μg/ml superoxide dismutase, 10^{-4} M 2,5-dimethylbenzoquinone added at dotted line; and (6) hepatocytes with 10^{-4} M NAPQI, 10^{-4} M 2,5-dimethylbenzoquinone added at dotted line.

ESR spectroscopy. No ESR spectra were obtained with reduction of NAPQI in aqueous solution by NADPH–cytochrome P-450 reductase and NADPH, rat hepatic microsomes and NADPH, or $NaBH_4$ (Fig. 6). Reduction of NAPQI by $NaBH_4$ in dimethyl sulfoxide gave a spectrum with G = 2.0033 and some hyperfine splitting but not enough resolution to assign splitting constants. The spectrum differs from that of the semiquinone imine of NAPQI generated by acetaminophen oxidation [10], but might be due to a polymer radical, since formation of polymeric material from NAPQI was apparent in dimethyl sulfoxide.

DISCUSSION

In aqueous solution, chemical reduction of NAPQI or reduction by rat hepatic microsomes and NADPH or by NADPH–cytochrome P-450 reductase and NADPH failed to give an ESR signal. Substrate inhibition by the high, millimolar, concentrations of NAPQI might account for the lack of a signal with NADPH–cytochrome P-450 reductase or further rapid reduction of $NAPQI^{\cdot-}$ to acetaminophen, a major product observed in these reactions. High drug concentrations are needed to obtain ESR spectra, probably due to disproportionation-comproportionation and stabilization of the free radical, thus permitting its detection [11]. This does not, however, explain the lack of a signal with chemical reduction of NAPQI in aqueous solution. NAPQI can be reduced chemically in dimethyl sulfoxide to form an ESR detectable free radical, although this may not be to the semiquinone free radical of NAPQI itself.

NAPQI was rapidly metabolized by NADPH–cytochrome P-450 reductase and had a K_m in the range 1.8 to 4 μM, somewhat lower than that of simple quinones [4]. Reduction of NAPQI under aerobic conditions produced acetaminophen as a major product, together with polymerized material, but apparently formed superoxide only slowly, actually preventing superoxide formation by other quinones. This is probably due to competitive inhibition of NADPH–cytochrome P-450 reductase by NAPQI. It is probable that reduction of NAPQI by NADPH–cytochrome P-450 reductase produces

Table 1. Effect of NAPQI on superoxide formation and on quinone-stimulated superoxide formation by NADPH–cytochrome P-450 reductase*

	$O_2^{\cdot-}$ formation (nmoles/min/mg)	
	without NAPQI	with 10^{-4} M NAPQI
Control	31.3 ± 4.8	0.0
10^{-4} M 2,5-Dimethylbenzoquinone	9465 ± 155	0.0

*Incubation conditions are described in the text. $O_2^{\cdot-}$ formation was measured as the reduction of acetylated cytochrome *c* at 550 nm at 37°, with and without superoxide dismutase, 66 μg/ml. Each value is the mean ± S.E.M. of three determinations.

Table 2. Effect of NAPQI on hepatocyte O_2 utilization and superoxide release*

	O_2 utilization (nmoles/min/10^6 cells)		Superoxide release (nmoles/min/10^6 cells)	
	without NAPQI	with 10^{-4} M NAPQI	without NAPQI	with 10^{-4} M NAPQI
Control	11.5 ± 0.6	9.2 ± 0.5†	0.3 ± 0.1	0.0 ± 0.0†
2,5-Dimethyl benzoquinone	20.0 ± 0.4	9.8 ± 0.6†	16.4 ± 0.3	0.0 ± 0.0†

*Incubation conditions are described in Fig. 5. Each value is the mean ± S.E.M. of three determinations.
†$P < 0.05$, compared to value in the absence of NAPQI.

the semiquinone imine radical which decays by mutual interaction to acetaminophen and NAPQI. Unlike simple quinones [8] NAPQI did not stimulate oxygen utilization by isolated hepatocytes and did not produce an increase in release of superoxide into the incubation medium, as measured by the reduction of acetylated cytochrome *c*.

NAPQI is the postulated toxic metabolite in acetaminophen overdose [1, 2]. NAPQI is cytotoxic to isolated hepatocytes [3] and, with A204 human rhabdomyosarcoma cells in culture, had an IC_{50} following 1 hr exposure of 2.0 μg/ml (unpublished observations). It is probable that NAPQI is metabolized by flavoenzyme in the liver to the semiquinone imine radical. We have shown that the semiquinone imine radical does not react rapidly with oxygen to form superoxide radical; therefore, it is unlikely that oxygen radicals are involved in the cytotoxic effect of NAPQI. The semiquinone imine radical might react directly with cellular constituents or initiate lipid peroxidation, although this has not been observed experimentally (unpublished observations). The major reaction of the radical seems to be further reduction to acetaminophen.

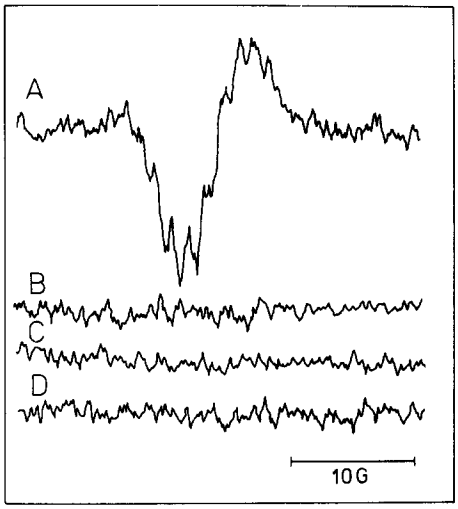

Fig. 6. ESR spectra formed by NAPQI. (A) 5 mM NAPQI in dimethyl sulfoxide reduced with a 75% stoichiometric amount of $NaBH_4$. (B) 5 mM NAPQI in 0.15 M KCl, 10 mM potassium phosphate, pH 7.0, reduced with a 75% stoichiometric amount of $NaBH_4$. (C) 5 mM NAPQI in 0.15 M KCl, 10 mM potassium phosphate, pH 7.0, with NADPH–cytochrome P-450 reductase, 1.4 units/ml, and 1 mM NADPH. (D) 5 mM NAPQI in 0.15 M KCl, 10 mM potassium phosphate, pH 7.0, with rat liver microsomes, 1 mg/ml and 1 mM NADPH.

In summary, ESR studies have shown that chemical reduction of NAPQI in dimethyl sulfoxide produces a free radical although this may not be the semiquinone imine free radical. No ESR evidence could be found for formation of a free radical from NAPQI in aqueous solution. NAPQI was rapidly metabolized by NADPH–cytochrome P-450 reductase but did not undergo significant redox cycling to form superoxide and showed substrate inhibition of enzyme activity. Furthermore, NAPQI did not stimulate oxygen utilization or superoxide release by isolated rat hepatocytes. Thus, if NAPQI is a toxic metabolite of acetaminophen, as expected, then both enzymatic and non-enzymatic reduction processes would represent protective mechanisms against cell injury caused by acetaminophen.

Acknowledgements—This work was supported by NIH Grants CA 33712 (G. P.) and GM 25418 (S. D. N.).

REFERENCES

1. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, M. Hashimoto and J. R. Mitchell, *Pharmacology* **12**, 251 (1974).
2. J. A. Hinson, L. R. Pohl, T. J. Monks and J. R. Gillette, *Life Sci.* **29**, 107 (1981).
3. D. C. Dahlin and S. D. Nelson, *J. med. Chem.* **25**, 885 (1982).
4. G. Powis and P. L. Appel, *Biochem. Pharmac.* **29**, 2567 (1980).
5. U. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
6. A. Azzi, C. Montecucco and C. Richter, *Biochem. biophys. Res. Commun.* **65**, 597 (1975).
7. D. J. Stewart and T. Inaba, *Biochem. Pharmac.* **28**, 461 (1979).
8. G. Powis, B. A. Svingen and P. L. Appel, *Molec. Pharmac.* **20**, 387 (1981).
9. E. Finkelstein, G. M. Rosen, E. J. Raukman and J. Paxton, *Molec. Pharmac.* **16**, 676 (1979).
10. P. A. West, L. S. Harman, P. D. Josephy and R. P. Mason, *Biochem. Pharmac.*, in press.
11. L. Michaelis, *Chem. Rev.* **16**, 243 (1935).