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CONVERSION OF 4-NITROQUINOLINE 1-OXIDE (4NQO) TO 4-HYDROXYAMINOQUINOLINE 1-OXIDE BY A DICUMAROL-RESISTANT HEPATIC 4NQO NITROREDUCTASE IN RATS AND MICE

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Abstract—The product formed from 4-nitroquinoline 1-oxide (4NQO), a potent carcinogen, by the action of mouse NADH:4NQO nitroreductase NR-1 was directly identified as 4-hydroxyaminoquinoline 1-oxide (4HAQO) by high performance liquid chromatography analyses in two systems. In liver cytosols from both male and female mice, NADH:4NQO nitroreductase was the predominant enzyme catalyzing the reduction of 4NQO. Rat liver cytosol catalyzed the conversion of 4NQO to either 4HAQO or a glutathione conjugate depending upon coenzyme or cosubstrate availability. Whereas NAD(P)H:quinone reductase (NAD(P)H:(quinone acceptor) oxidoreductase; DT diaphorase; EC 1.6.99.2) was the predominant 4NQO reductase present in liver cytosol from Sprague–Dawley rats, dicumarol-resistant NADH:4NQO nitroreductase specific activities were comparable with those of mouse liver cytosols. A 4NQO nitroreductase from rat liver cytosol was separated from NAD(P)H:quinone reductase chromatographically and shown to have a strong preference for NADH and to be insensitive to inhibition by dicumarol.

4-Nitroquinoline 1-oxide (4NQO†) may be activated by nitroreduction to 4-hydroxyaminoquinoline 1-oxide (4HAQO), a proximate carcinogen, or conjugatively inactivated by glutathione transferases (EC 2.5.1.18) [1–6]. The structures of 4NQO, 4HAQO, and the glutathione conjugate are shown in Fig. 1. In studies on rat liver, Kato *et al.* [7] and Sugimura *et al.* [8] showed that the reduction of 4NQO to 4HAQO is catalyzed by NAD(P)H:quinone reductase, an enzyme characterized by its sensitivity to inhibition by dicumarol and its dual pyridine nucleotide specificity [9]. These studies [7, 8] yielded no evidence for the presence of a dicumarol-resistant NADH:4NQO nitroreductase.

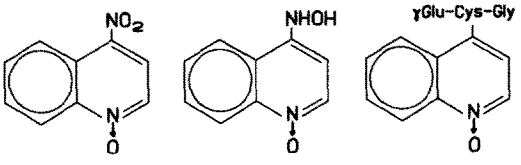

Fig. 1. Structures of 4NQO and the products of its nitroreduction and conjugation.

We recently reported that the predominant 4NQO reductase in liver cytosols from female Swiss mice is not NAD(P)H:quinone reductase but a dicumarol-resistant enzyme [10]. This enzyme, designated NR-1, exhibits a strong preference for NADH rather than NADPH and oxidizes 2 molecules of NADH per molecule of 4NQO reduced [10]. NAD(P)H:quinone reductase accounts for a much lesser portion of the cytosolic 4NQO nitroreductase activity. The newly described 4NQO nitroreductase differs from NAD(P)H:quinone reductase not only in its dicumarol insensitivity and its preference for NADH but also in having a much lower K_m for 4NQO and a much higher molecular weight [10]. The goals of the present investigation were to determine whether the product of the NADH-dependent reduction of 4NQO by the mouse nitroreductase NR-1 was 4HAQO, as shown for NAD(P)H:quinone reductase [8], and to determine whether rat liver cytosol might also contain an NADH:4NQO nitroreductase distinct from NAD(P)H:quinone reductase.

MATERIALS AND METHODS

Chemicals and livers. NADH, NADPH, bovine serum albumin, and dicumarol were purchased from the Sigma Chemical Co. (St. Louis, MO). 4NQO and 4HAQO were from the Aldrich Chemical Co. (Milwaukee, WI) and American Tokyo Kasei, Inc. (Portland, OR), respectively. Livers from male and female Swiss–Webster mice and Sprague–Dawley rats were obtained from Pel-Freez Biologicals (Rogers, AR). The livers had been individually frozen in liquid nitrogen immediately upon excision and were stored at −70°.

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† Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; and 4HAQO, 4-hydroxyaminoquinoline 1-oxide.

Preparation of liver cytosols and 4NQO nitroreductases. Liver portions from six animals per group were pooled and homogenized at 0–4° in 0.25 M sucrose (3 mL/g liver), and cytosol fractions were prepared as described previously [11]. Protein concentrations were determined by the method of Lowry *et al.* [12] with bovine serum albumin as a standard. The partial purification and characterization of nitroreductases from liver cytosols of female Swiss mice have been described previously [10]. These enzymes were designated NR-1 and NR-2 (NAD(P)H:quinone reductase) according to their order of elution from a column of Sephadex G-150. The NR-1 and NR-2 preparations used in this study catalyzed the 4NQO-dependent oxidation of NADH with specific activities of 38 and 23 nmol/min/mg protein, respectively.

Preparation of 4NQO and 4HAQO stock solutions. 4NQO (10 mM) was dissolved in 95% ethanol. 4HAQO stock solutions (1 mM) were prepared in a 1:1 (v/v) mixture of 0.1 M sodium acetate, pH 5.0, and methanol [13].

Spectrophotometric assay of nitroreductase activities. Activity was measured in a 1-mL system at 25° containing 0.1 mM 4NQO, 0.06 mM NADH or NADPH, and an appropriate amount of enzyme source, in 50 mM potassium phosphate, pH 6.5 [10]. The initial rate of oxidation of the pyridine nucleotide was monitored at 340 nm and corrected for the rate observed in the absence of 4NQO. The results are expressed as nanomoles NADH or NADPH oxidized per minute.

High performance liquid chromatography (HPLC) analyses. A Waters (Division of Millipore, Milford, MA) model 625 liquid chromatography system was used that included a Rheodyne model 9125 injector and a Waters model 484 absorbance detector. A 3.9 × 300 mm Waters μBondapak C-18 column was operated at ambient temperature, and the absorbance of the effluent was monitored at 340 nm. Two methods were used. In Method 1, the flow rate was 0.5 mL/min and the mobile phase consisted of a gradient from 65% Solvent A and 35% Solvent B to 45% Solvent A and 55% Solvent B in 25 min, where Solvent A was 25 mM sodium acetate in 25 mM triethylamine, adjusted to pH 5.00 with acetic acid, and Solvent B was methanol. In Method 2, the flow rate was 1 mL/min and the mobile phase was composed of 0.1% (v/v) trifluoroacetic acid containing either 10% (v/v) acetonitrile (Solvent A) or 60% acetonitrile (Solvent B). The column was equilibrated with Solvent A and developed with a linear gradient to Solvent B in 8 min, followed by Solvent B. A Baseline 815 software package (Dynamic Solutions, Ventura, CA) was used to generate gradients and to acquire and process data.

Chromatographic separation of 4NQO nitroreductases. Cytosol (2.75 mL; equivalent to 1 g of liver), freshly prepared from pooled liver portions from three male Sprague–Dawley rats, was supplemented with dithiothreitol (0.1 mM) and applied to a 4-mL column of DEAE-cellulose (Whatman DE-52) equilibrated and developed with 10 mM potassium phosphate, 0.2 mM dithiothreitol, at pH 6.80 (Buffer A) at 4°. The unretained nitroreductase activity was applied in 4 mL to a 1 × 10 cm column of S-Sepharose (fast flow) operated at 4° in Buffer A at a flow rate of 26 mL/hr. Fractions (2 mL) were collected. After 53 mL the eluent was changed to 1 M KCl in Buffer A. The absorbance of the effluent at 280 nm was measured by use of a Pharmacia UV-2 monitor. NADH:4NQO nitroreductase activity was measured in the fractions. Activities were corrected for blanks that contained ethanol in place of 4NQO.

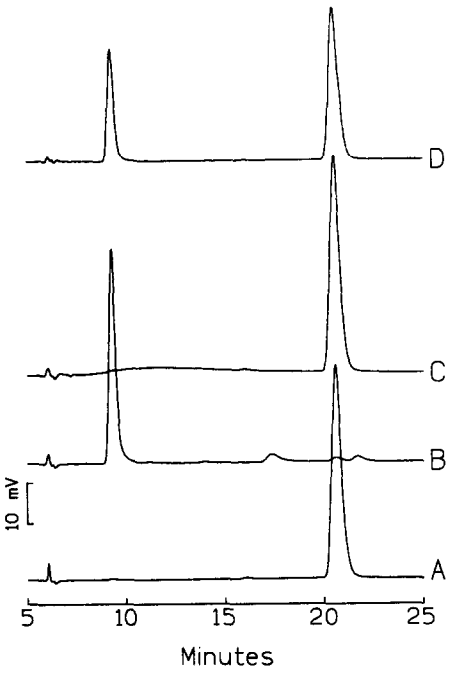

Fig. 2. HPLC analysis by Method 1 of the product of 4NQO reduction by murine nitroreductase NR-1. The enzyme was partially purified from mouse liver. The elution profile of a 20-μL sample taken after 20 min of incubation of 0.1 mM 4NQO with 60 μM NADH and nitroreductase NR-1 (80 μg protein/mL) is shown (D), in comparison with the elution profiles obtained with 2 nmol 4NQO (A) and 2 nmol 4HAQO (B). Chromatogram C shows the elution profile of a 20-μL sample from an incubation in which only the enzyme was omitted. The absorbance at 340 nm was monitored. The conditions of incubation and analysis are described in the text.

RESULTS AND DISCUSSION

Identification of the product of 4NQO reduction by the NADH:4NQO reductase. Our previous studies had not identified the product of the reduction of 4NQO by the mouse NADH:4NQO nitroreductase, NR-1, although the stoichiometry of 2 molecules of NADH per molecule of 4NQO was consistent with the production of 4HAQO [10]. In the current investigation, two analytical HPLC methods, described in Materials and Methods, were developed for direct identification of the product of the enzymic reduction of 4NQO. Product was generated by incubating 0.1 mM 4NQO and 0.06 mM NADH in 50 mM potassium phosphate, pH 6.5, at room

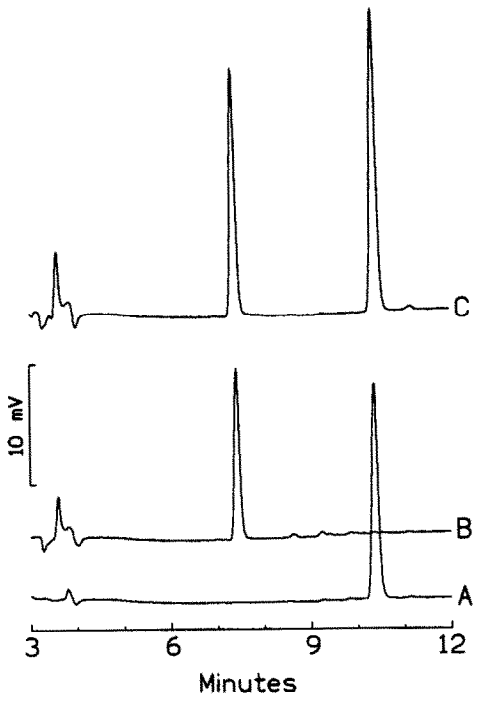

Fig. 3. HPLC analysis by Method 2 of the product formed from 4NQO by murine nitroreductase NR-1. The HPLC elution profile obtained after 12 min of incubation of 0.1 mM 4NQO with 60 μM NADH and nitroreductase NR-1 (80 μg protein/mL) is shown (C), in comparison with the elution profiles obtained with 0.4 nmol 4NQO (A) and 0.33 nmol 4HAQO (B). A portion of the incubation mixture was added to 1.5 vol. of methanol, and 20 μL was used for analysis. The absorbance at 340 nm was monitored. The conditions of incubation and analysis are described in the text.

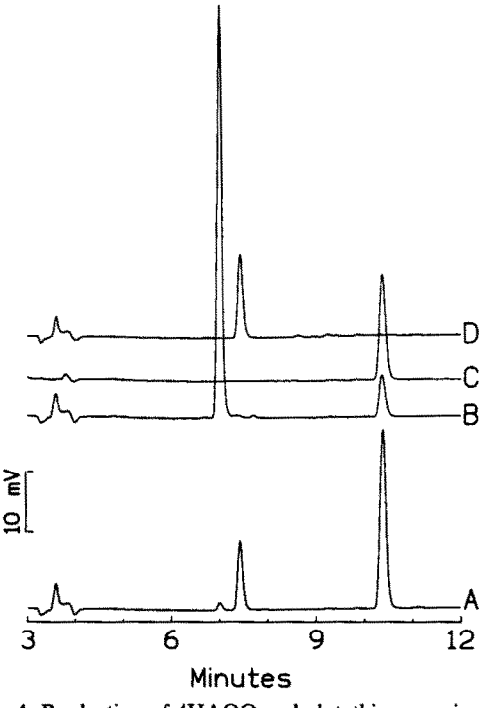

Fig. 4. Production of 4HAQO and glutathione conjugate from 4NQO by rat liver cytosol. 4NQO (0.1 mM) and liver cytosol from female Sprague–Dawley rats (0.24 mg protein/mL) were incubated for 2 min with either 60 μM NADH (A) or 1 mM glutathione (B), and HPLC analyses were performed by Method 2. The elution profiles of 4NQO (C) and 4HAQO (D) are shown for comparison. The experimental conditions were as described in the legend to Fig. 3 and in the text.

temperature with nitroreductase NR-1 (0.08 mg protein/mL). The incubation conditions were chosen to correspond to those used in the spectrophotometric 4NQO nitroreductase assay system. In this system, the production of 4HAQO is limited not by the concentration of 4NQO but by the availability of NADH.

The results of HPLC analyses by Method 1 are shown in Fig. 2. Commercially obtained 4NQO and 4HAQO were used as standards. Their elution profiles are shown as Chromatograms A (4NQO) amd B (4HAQO). The retention time of 4NQO was 20.5 min and that of 4HAQO was 9.2 min in this system. Chromatography of a 20-μL sample after 20 min of incubation of 4NQO with NADH and nitroreductase NR-1 gave the results shown as Chromatogram D, consistent with 4HAQO as the product of the enzymic reaction. No measurable production of 4HAQO occurred in an incubation from which the enzyme was omitted (Chromatogram C).

Figure 3 shows the results of HPLC analyses by Method 2. Chromatograms A and B show elution profiles of 4NQO and 4HAQO, respectively. In this system the retention times were 10.4 min for 4NQO and 7.4 min for 4HAQO. Incubations were performed as described above and aliquots were removed periodically and added to 1.5 vol. of methanol to inactivate the enzyme. Chromatogram C in Fig. 3 shows the elution profile of a 20-μL sample derived from a 12-min incubation. The reaction was 97% complete, with only 3% further change in 4HAQO and 4NQO concentrations observed after 30 and 70 min of incubation. The retention time of the product of 4NQO reduction by NR-1 was identical to that of 4HAQO, thus confirming the identification of 4HAQO as the product. Incubation of the mouse liver NAD(P)H:quinone reductase, nitroreductase NR-2 (0.17 mg protein/mL), with NADH and 4NQO for 30 min under the conditions described above also yielded 4HAQO as the only product (data not shown). No 4HAQO was produced in incubations from which enzyme was omitted. Thus, HPLC analysis by two methods (i.e. by gradient elution in two distinct mobile phase systems) yielded direct identification of 4HAQO as the product of the NADH-dependent reduction of 4NQO by nitroreductase NR-1.

Metabolism of 4NQO by rat liver cytosol. Two products were observed when cytosol prepared from pooled liver portions from six female Sprague–

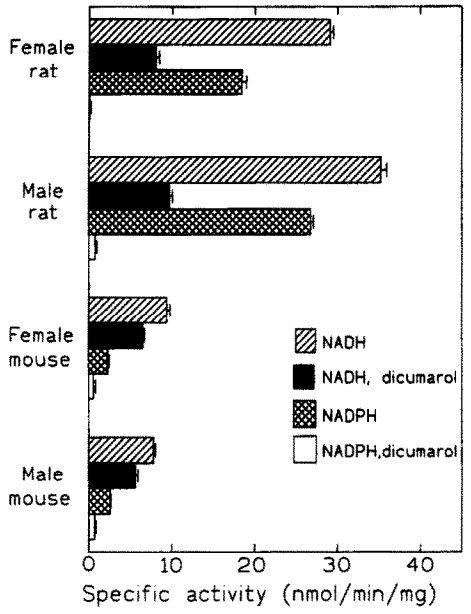

Fig. 5. NADH- and NADPH-dependent 4NQO nitroreductase activities of female and male rat and mouse liver cytosol preparations. The activities, measured spectrophotometrically in the absence of dicumarol and in the presence of 15 μM dicumarol, are expressed as nanomoles of pyridine nucleotide oxidized per minute per milligram of cytosol protein. Each value is the mean ± SEM of at least three measurements.

Dawley rats was incubated with 4NQO and NADH under conditions identical to those described above. After 2 min of incubation of 4NQO, NADH, and rat liver cytosol (0.24 mg protein/mL), HPLC analysis by Method 2 gave the elution profile shown in Fig. 4, Chromatogram A. The elution profiles of 4NQO (C) and 4HAQO (D) are shown for comparison. The retention time of 7.4 min for the major product from the incubation corresponded to that of 4HAQO while a lesser component appeared at 7.0 min. The appearance of 4HAQO as the product of 4NQO nitroreduction by rat liver cytosol is in accord with the published results of Sugimura *et al.* [8]. The enzymic conjugation of 4NQO with glutathione, the isocratic HPLC analysis of the glutathione conjugate, and the structural characterization of the conjugate as 4-(glutathion-*S*-yl)-quinoline 1-oxide have been reported previously [5, 14]. It has also been shown that the conjugation of 4NQO with glutathione at pH 6.5 is almost entirely dependent upon enzymic catalysis and that rat liver cytosol contains abundant glutathione transferase activity towards 4NQO [5]. To explore the possibility that the component exhibiting a retention time of 7.0 min might be a product of the conjugation of 4NQO with endogenous glutathione present in the cytosol, 4NQO was incubated with rat liver cytosol under the conditions described above but with the substitution of glutathione (1 mM) for the NADH. HPLC analysis revealed that after only 2 min of incubation 81% of the 4NQO had been consumed and the area of the peak at 7.0 min had increased 54-fold (Fig. 4, Chromatogram B), confirming that this peak represented a glutathione conjugate formed from 4NQO. Thus, rat liver cytosol converted 4NQO either predominantly to 4HAQO (Chromatogram A) or to a glutathione conjugate (Chromatogram B) depending upon the nature of the coenzyme provided.

Dicumarol-sensitive and dicumarol-resistant NADH:4NQO and NADPH:4NQO nitroreductase activities of liver cytosols from female and male rats and mice. The cytosol fractions prepared from pooled liver portions from six animals per group, as described above, had protein concentrations of 23.7 mg/mL (female rats), 22.9 mg/mL (male rats), 21.1 mg/mL (female mice), 21.9 mg/mL (male mice). The cytosol fractions were assayed spectrophotometrically for 4NQO nitroreductase activity with NADH and with NADPH as electron donors in the absence of dicumarol and in the presence of 15 μM dicumarol to distinguish the dicumarol-sensitive activity of NAD(P)H:quinone reductase from dicumarol-resistant activity. The results are shown in Fig. 5. In the absence of dicumarol and with either NADPH or NADH as the electron donor, the rats exhibited much higher hepatic 4NQO nitroreductase activity than did the mice. Measurements in the presence of dicumarol showed that this difference was due to higher activities of NAD(P)H:quinone reductase in rat liver. In both species and genders there was substantial dicumarol-resistant activity only when NADH served as the electron donor. This hepatic dicumarol-resistant NADH:4NQO nitroreductase activity, which we observed previously in female mice and purified as 4NQO nitroreductase NR-1 [10], was found in similar amounts in male mice and in similar or somewhat greater amounts in both male and female rats.

Chromatographic separation of two distinct rat liver cytosolic enzymes catalyzing the pyridine nucleotide-dependent nitroreduction of 4NQO. Chromatography of a rat liver cytosol preparation on S-Sepharose, as described in Materials and Methods, yielded two peaks of NADH:4NQO nitroreductase activity, as shown in Fig. 6. The fractions composing each peak were pooled and examined for pyridine nucleotide preference and dicumarol sensitivity. The nitroreductase that was unretained by S-Sepharose was 100% as active with NADPH as with NADH and was 99% inhibited by 15 μM dicumarol, as would be expected for NAD(P)H:quinone reductase. The second peak of 4NQO nitroreductase activity was clearly distinct from NAD(P)H:quinone reductase, as it was found to be completely resistant to inhibition by 15 μM dicumarol and to exhibit a strong preference for NADH, being only 15% as active with NADPH as the electron donor. Clearly both of these enzymes contribute to the nitroreduction of 4NQO by rodent liver cytosols with the main difference in 4NQO nitroreduction by rat and mouse liver cytosols being the greater rate of catalysis by NAD(P)H:quinone reductase in rat liver in contrast to the predominance of the dicumarol-resistant NADH:4NQO nitroreductase in mouse liver.

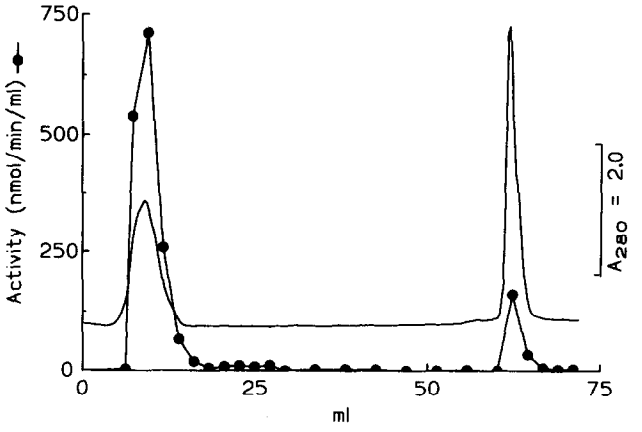

Fig. 6. Chromatographic separation of two 4NQO nitroreductases of rat liver cytosol. A preparation equivalent to 1 g of liver from male Sprague–Dawley rats was chromatographed on a 1 × 10 cm column of S-Sepharose as described in the text. The 4NQO nitroreductase activities of the fractions were measured spectrophotometrically with NADH as the electron donor. The activities of the pooled peak fractions with NADPH and the effects of dicumarol are described in the text.

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REFERENCES

1. Ito N, *In vivo* carcinogenesis of 4-nitroquinoline 1-oxide and related compounds. In: *Carcinogenesis—A Comprehensive Survey* (Ed. Sugimura T), Vol. 6, pp. 117–153. Raven Press, New York, 1981.
2. Matsushima T and Sugimura T, Chemistry and biological actions of 4-nitroquinoline 1-oxide. Metabolism. *Recent Results Cancer Res* **34**: 53–60, 1971.
3. Tada M, In: *Carcinogenesis—A Comprehensive Survey* (Ed. Sugimura T), Vol. 6, pp. 25–45. Raven Press, New York, 1981.
4. Al-Kassab S, Boyland E and Williams K, An enzyme from rat liver catalysing conjugations with glutathione. 2. Replacement of nitro groups. *Biochem J* **87**: 4–9, 1963.
5. Stanley JS and Benson AM, The conjugation of 4-nitroquinoline 1-oxide, a potent carcinogen, by mammalian glutathione transferases. *Biochem J* **256**: 303–306, 1988.
6. Aceto A, Di Ilio C, Lo Bello M, Bucciarelli T, Angelucci S and Federici G, Differential activity of human, rat, mouse, and bacteria glutathione transferase isoenzymes towards 4-nitroquinoline 1-oxide. *Carcinogenesis* **11**: 2267–2269, 1990.
7. Kato R, Takahashi A and Oshima T, Characteristics of nitro reduction of the carcinogenic agent, 4-nitroquinoline 1-oxide. *Biochem Pharmacol* **19**: 45–55, 1970.
8. Sugimura T, Okabe K and Nagao M, The metabolism of 4-nitroquinoline 1-oxide, a carcinogen. III. An enzyme catalyzing the conversion of 4-nitroquinoline 1-oxide to 4-hydroxyaminoquinoline 1-oxide in rat liver and hepatomas. *Cancer Res* **26**: 1717–1721, 1966.
9. Ernster L, DT diaphorase. *Methods Enzymol* **10**: 309–317, 1967.
10. Stanley JS, York JL and Benson AM, Nitroreductases and glutathione transferases that act on 4-nitroquinoline 1-oxide and their differential induction by butylated hydroxyanisole in mice. *Cancer Res* **52**: 58–63, 1992.
11. Benson AM, Hunkeler MJ and Talalay P, Increase of NAD(P)H:quinone reductase by dietary antioxidants: Possible role in protecting against carcinogenesis and toxicity. *Proc Natl Acad Sci USA* **77**: 5216–5220, 1980.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Kano K, Uno B, Kaida N, Zhang Z-X, Kubota T, Takahashi K and Kawazoe Y, Voltammetric and spectroscopic studies on the carcinogen 4-(hydroxyamino)quinoline *N*-oxide and its analogues. *Chem Pharm Bull (Tokyo)* **35**: 1702–1714, 1987.
14. Stanley JS, Lay JO Jr, Miller DW and DeLuca DC, Identification of the glutathione conjugate of 4-nitroquinoline 1-oxide formed in the reaction catalyzed by murine glutathione transferases. *Carcinogenesis* **10**: 587–591, 1989.