Biochemical Pharmacology, Vol. 33, No. 3, pp. 499–502, 1984.
Printed in Great Britain.

0006–2952/84 $3.00 + 0.00
Pergamon Press Ltd.

Effect of uroporphyrin on the spectral measurement of cytochrome P450

(*Received* 5 *March* 1983; *accepted* 3 *August* 1983)

Porphyria cutanea tarda (PCT)* is a chronic hepatic porphyria that occurs in man and other mammals and resembles a toxic hepatic porphyria that occurs in humans and animals exposed to hexachlorobenzene or certain halogenated biphenyls [1, 2]. In these porphyrias, uro- and heptacarboxyl porphyrins accumulate in the liver due to decreased activity of hepatic uroporphyrinogen decarboxylase [3–5]. It has been reported that patients with PCT have greatly elevated concentrations of hepatic cytochrome P450, although no corresponding increases in the rates of drug oxidation catalyzed by cytochrome P450 were observed [6–8]. Large increases in hepatic cytochrome P450 have also been reported in rats treated with hexachlorobenzene [9]. Here we report that such increases are artifacts arising from the method used to measure cytochrome P-450 in these studies. When cytochrome P-450 is measured as the dithionite-reduced CO complex by difference spectroscopy in homogenates of human liver, the method of Schoene *et al.* [10] is used to eliminate interference from the reduced hemoglobin–CO complex. In this paper, we show that, when the method of Schoene *et al.* [10] is applied to homogenates of human, rat, or chick liver which contain large amounts of uroporphyrin, a false apparent increase in cytochrome P-450 is detected. The apparent increase is an artifact caused by the dithionite reduction of uroporphyrin to dihydrouroporphyrin. Portions of this work have appeared in abstract form [11, 12].

Methods

Homogenates of human liver (10–15 mg wet liver/ml) were prepared from biopsy specimens in 0.1 M sodium phosphate buffer (pH 7.4). Twenty percent (w/v) homogenates of normal rat liver were prepared in 0.25 M sucrose–0.02 M Tris–Cl (pH 7.4)–0.09 mM butylated hydroxytoluene and diluted 1:80 (v/v) with 0.1 M sodium phosphate (pH 7.4) before use.

Cultures of chick embryo liver cells and homogenates of these cells were prepared as described previously [13]. After the first 18 hr in culture, cells were rinsed with fresh medium and exposed for an additional 20 hr to chemicals dissolved in dimethyl sulfoxide (DMSO) or to DMSO alone.

Cytochrome P-450 was assayed spectrophotometrically in liver homogenates by two methods. Method I is that of Schoene *et al.* [10], in which the entire homogenate is bubbled with CO for 20–30 sec, split into two cuvettes, the baseline recorded, and the contents of the sample cuvette reduced with dithionite. After 2 min the difference spectrum is recorded. Method II is that of Omura and Sato [14] in which the entire homogenate is first reduced with dithionite, then split into two cuvettes, the baseline recorded, the sample cuvette bubbled with CO, and the difference spectrum recorded. Note, that in Method I, dithionite is added to the sample cuvette only; thus, the spectra obtained with Method I may be susceptible to interference from compounds that are reducible by dithionite.

Concentrations of urinary porphyrins, porphyrin precursors and liver iron in human samples were measured as described [15]. Liver porphyrins were extracted into 1 M perchloric acid–methanol (1:1, v/v) and measured by the method of Grandchamp *et al.* [16]. Antipyrine metabolism studies in humans were done as in Ref. 17 under a protocol approved by the Human Investigation Committee of the Dartmouth-Hitchcock Medical Center. Antipyrine was measured [18] in samples of saliva obtained 3, 6, 9, 12 and 24 hr after ingestion of a solution of antipyrine (18 mg/kg body wt).

Protein was measured by the method of Lowry *et al.* [19] using bovine serum albumin as standard.

Uro-, copro-, or protoporphyrin (all from Porphyrin Products, Logan, UT) were dissolved in DMSO (200 μg/ml). Portions were diluted in HCl for quantitative determination by absorption spectrophotometry [20].

Patients. Three male patients, aged 53, 58 and 68 years, with typical clinical and biochemical features of PCT were studied. At the time of biopsy, all were excreting large amounts of urinary uroporphyrin (1.1, 1.3 and 1.5 mg/g creatinine; normal = 0–60 μg/g creatinine), but were excreting normal amounts of δ-aminolevulinate, porphobilinogen and coproporphyrin. All livers contained high concentrations of porphyrin (0.3, 0.47 and 1.27 nmoles/mg protein), most of which was uroporphyrin. The liver iron concentrations of the patients were elevated (193, 306 and 458 μg iron/100 mg dry liver weight, normal range 34–136 μg iron/100 mg dry weight) [21]. Histological examination showed alcoholic hepatitis and cirrhosis in one patient, mild portal fibrosis and chronic hepatitis in the second, and mild portal fibrosis in the third. 'Normal' liver tissue was obtained from patients in the course of diagnostic studies and contained normal amounts of iron and no detectable uroporphyrin.

Result and Discussion

Figure 1A shows difference spectra of homogenates of liver from one of the patients with PCT and from a normal patient, using Method I. In agreement with earlier reports [6–8], the liver homogenate from this PCT patient appeared to have a much higher concentration of cytochrome P-450 than the normal patient. Liver homogenates from the other two PCT patients also appeared to contain high concentrations of cytochrome P-450. There were two unusual features of the spectra of homogenates from the PCT patients: a shift in the wavelength of the absorbance peak (from 448–451 nm in controls to 442–444 nm in PCT patients), and an increase in the time required for maximum reduction by dithionite (2 min for controls, greater than 5 min for PCT patients). Despite the apparent spectral increase in cytochrome P-450, clearance of antipyrine *in vivo* in two of the PCT patients (1.63, 1.65 l/hr) was less than normal (3.77 ± 1.65 l/hr, mean ± S.D.). Others have found normal rates of oxidation of benz[*a*]pyrene and aminopyrine by homogenates of PCT liver [6–8].

Since livers of patients with PCT accumulate uroporphyrin, we studied the effect of exogenous uroporphyrin on the spectral measurement of cytochrome P-450 by Method I. We found that when uroporphyrin was added to homogenate of rat liver, the spectrum showed a large increase in absorbance and a shift of the peak from 449 to 444 nm (Fig. 1B).

To establish that *endogenously* produced uroporphyrin could similarly affect the difference spectra obtained with

* Abbreviations: PCT, porphyria cutanea tarda; DMSO, dimethyl sulfoxide; PIA, propylisopropylacetamide; and PBB, 2,4,5,3′,4′-pentabromobiphenyl.

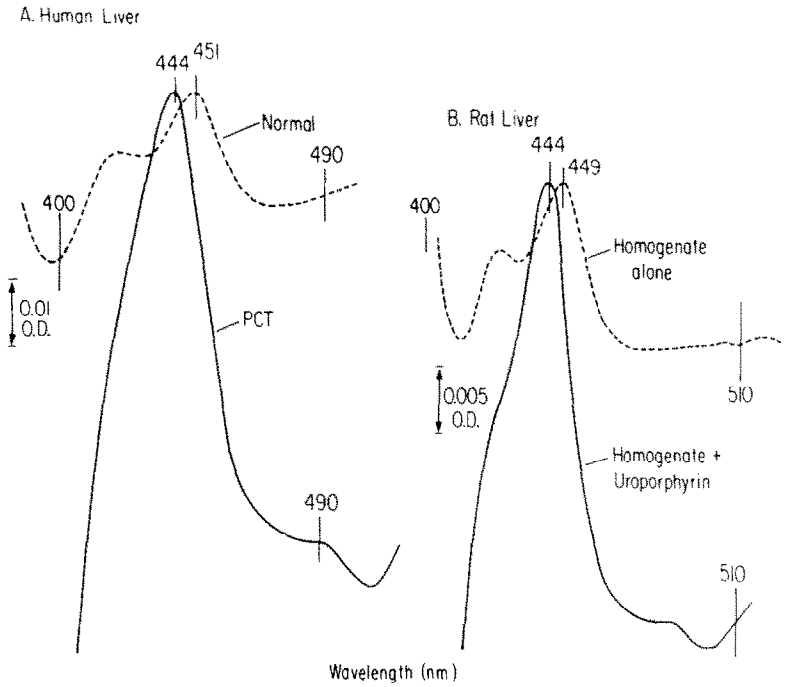

Fig. 1. Difference spectra of human and rat liver homogenate obtained with Method I. Homogenates were prepared as described in Methods. (A) Human liver, normal and PCT: homogenates contained 2.3 mg protein/ml. The normal homogenate contained no detectable porphyrin, whereas the PCT homogenate contained 695 pmoles uroporphyrin/ml. (B) Rat liver with and without added uroporphyrin: homogenates contained 0.5 mg protein/ml. Uroporphyrin I was added to the rat liver homogenate to a final concentration of 970 pmoles/ml. When Method I was used on homogenates of rat liver, NADH (0.4 mM) was added before gassing with CO to eliminate interference from cytochrome b_5[22].

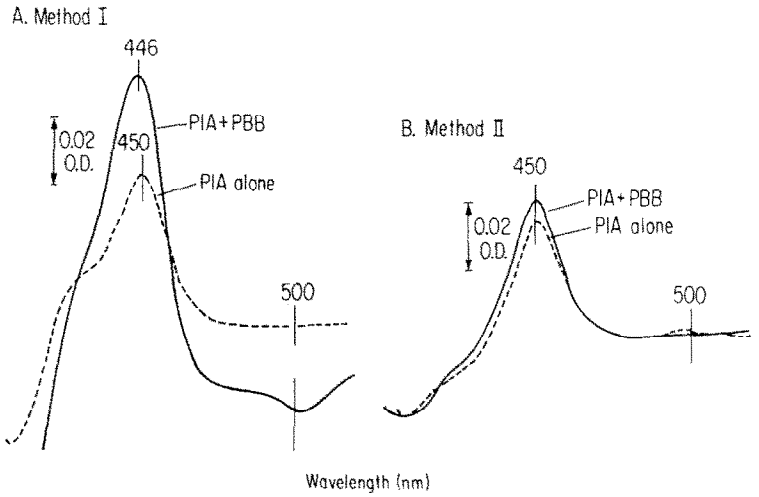

Fig. 2. Effect of accumulated uroporphyrin on difference spectra of cultured chick embryo liver cells, measured by Method I (A) vs Method II (B). Cultures were exposed for 20 hr to solvent alone (DMSO, 2 μm/ml), to propylisopropylacetamide (PIA, 20 μg/ml), or to PIA plus 2,4,5,3′,4′-pentabromobiphenyl (PBB, 1 μg/ml). Difference spectra were obtained on 8700 *g* supernatant fractions of the cells prepared as described previously [23]. Porphyrin accumulations (pmoles/mg protein) were as follows: DMSO alone, 49; PIA, 97; and PIA + PBB, 1150. Twenty-seven percent of porphyrins in control or PIA-treated cells were uroporphyrin; in PIA + PBB-treated cells, 87% was uroporphyrin.

Method I, we studied cultures of chick embryo hepatocytes in which cytochrome P-450 was induced by propylisopropylacetamide (PIA) [23]. The simultaneous exposure of the cells to 2,4,5,3′,4′-pentabromobiphenyl (PBB) and PIA results in the accumulation of large amounts of uroporphyrin [24]. Figure 2A shows that, with Method I, cells treated with both PIA and PBB appeared to contain twice as much cytochrome P-450 as cells treated with PIA alone. However, when Method II was used to measure cytochrome P-450 in the same samples, no appreciable difference was observed (Fig. 2B). Furthermore, the shift in maximum wavelength obtained with Method I was not seen when Method II was used. These results show that uroporphyrin, even when endogenously produced, will interfere with the spectral measurement of cytochrome P-450 when measured using Method I but not when using Method II.

Figure 3A shows the changes in the absorbance spectrum of uroporphyrin following reduction with dithionite. The decrease of the Soret peak at 397 nm and the appearance of a peak at 440 nm are due to the reduction of uroporphyrin to dihydrouroporphyrin [25]. The reduced minus oxidized difference spectrum of uroporphyrin (Fig. 3B) also has an absorbance peak at 440 nm. Although the extinction coefficients of uroporphyrin (at 397 nm) and dihydrouroporphyrin (at 440 nm) are similar [25], the increase in absorbance at 440 nm is less than the decrease at 397 nm when uroporphyrin is reduced by dithionite, because further reduction of dihydrouroporphyrin occurs [25]. These results explain why the presence of uroporphyrin gives a spuriously high value of cytochrome P-450 when measured using Method I.

Coproporphyrin can also be reduced by dithionite to dihydrocoproporphyrin which absorbs at 436 nm [25]. However, we found that the height of the peak at 436 nm due to dihydrocoproporphyrin was only about 10% of that given by the reduction of an equivalent concentration of uroporphyrin. Spectra of protoporphyrin, either alone or in the presence of rat liver homogenates, were unchanged by addition of dithionite. These results explain why false increases in hepatic cytochrome P-450 have not been observed in variegate or protoporphyria in which copro- and proto-, but not uroporphyrin, accumulate [8].

In summary, the presence of high concentrations of uroporphyrin in liver homogenates, such as in human PCT, interfered with the measurement of cytochrome P-450 when Method I was used. In Method I, homogenates were treated with CO, and only the sample cuvette was reduced with dithionite (Fig. 1). The erroneous result obtained with Method I was due to the reduction of uroporphyrin to dihydrouroporphyrin which absorbs at 440 nm (Fig. 3). A false increase in cytochrome P-450 was not detected when Method II was used in which both cuvettes were reduced with dithionite and only the sample cuvette was gassed with CO (Fig. 2). Unfortunately, Method II cannot be used on samples that contain hemoglobin. However, if conditions could be devised whereby cytochrome P-450 becomes fully reduced without reducing uroporphyrin (e.g. enzymatic reduction via NADPH–cytochrome P-450 reductase), Method I could be used for accurate measurements of cytochrome P-450 in samples from PCT patients. Our results with homogenates containing uroporphyrin illustrate a pitfall in the measurement of cytochrome P-450 and suggest that caution is needed in the measurement of cytochrome P-450 in the presence of chemicals reducible by dithionite.

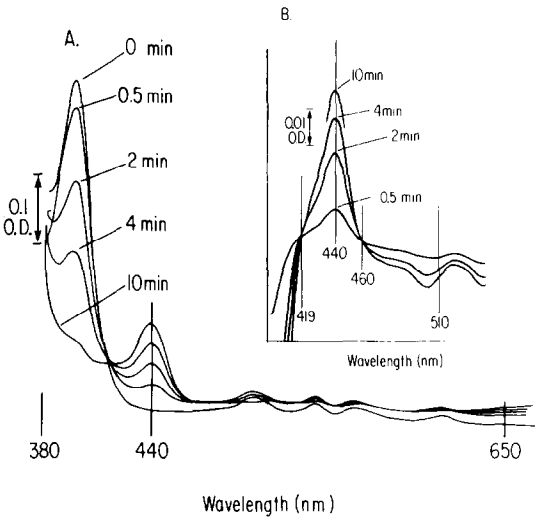

Fig. 3. Progress of spectral change during reduction of uroporphyrin by dithionite. Uroporphyrin (1 μM) was in 0.1 M sodium phosphate buffer, pH 7.4. (A) Absorbance spectrum: the sample cuvette contained uroporphyrin and the reference cuvette contained buffer only. Dithionite was added to both cuvettes to initiate reduction, and spectra were recorded at the times shown. (B) Difference spectrum: uroporphyrin was in both cuvettes. Dithionite was added to the sample cuvette to initiate reduction, and spectra were recorded at the times shown.

Acknowledgements—We thank Joanne Pomeroy for technical assistance and E. Lucile Smith for her valuable advice. Purified pentabromobiophenyl was a gift from S. Aust; PIA was a gift from Hoffmann-LaRoche. Supported by research funds from the U.S. Veterans Administration and by a grant (CA 25012) from NIH.

Hepatology/Metabolism Laboratory
VA Medical Center
White River Junction, VT 05001, and
Departments of Medicine and Biochemistry
Dartmouth Medical School
Hanover, NH 03755, U.S.A.

HERBERT L. BONKOVSKY*
JOHN F. HEALEY
WILLIAM J. BEMENT
PETER R. SINCLAIR
JACQUELINE F. SINCLAIR
STEVEN I. SHEDLOFSKY

REFERENCES

1. H. L. Bonkowsky, in *Hepatology: A Textbook of Liver Disease* (Eds. D. Zakim and T. D. Boyer), p. 351. Saunders, Philadelphia (1982).
2. G. H. Elder, *Handbk. exp. Pharmac.* **44**, 157 (1978).
3. J. P. Kushner, A. J. Barbuto and G. R. Lee, *J. clin. Invest.* **58**, 1089 (1976).
4. A. V. Benedetto, J. P. Kushner and J. S. Taylor, *New Engl. J. Med.* **298**, 358 (1978).
5. G. H. Elder, D. M. Sheppard, R. E. de Salamanca and A. Olmos, *Clin. Sci.* **58**, 477 (1980).
6. N. Pimstone, G. Blekkenhorst and L. Eales, *Enzyme* **16**, 354 (1973).
7. G. Blekkenhorst, N. R. Pimstone and L. Eales, in *Porphyrins in Human Disease* (Ed. M. Doss), p. 299. Karger, Basel (1976).
8. G. H. Blekkenhorst, N. R. Pimstone, B. L. Webber and L. Eales, *Ann. clin. Res.* **8** (Suppl. 17), 108 (1976).

* Address all correspondence to: Herbert L. Bonkovsky, M. D., Division of Digestive Diseases, 405/151, VA Medical and Regional Office Centre, White River Junction, VT 05001 U.S.A.

9. G. H. Blekkenhorst, L. Eales and N. R. Pimstone, *Clin. Sci. molec. Med.* **55**, 461 (1978).
10. B. Schoene, R. A. Fleischmann, H. Remmer and H. F. von Oldershausen, *Eur. J. clin. Pharmac.* **4**, 65 (1972).
11. H. Bonkowsky, J. Pomeroy, J. Healey, P. Sinclair and J. Sinclair, *Gastroenterology* **80**, 1328 (1981).
12. H. Bonkowsky, P. Sinclair, J. Bement, J. Healey, J. Sinclair and S. Shedlofsky, *Hepatology* **2**, 732 (1982).
13. J. F. Sinclair, P. R. Sinclair, J. F. Healey, E. L. Smith and H. L. Bonkowsky, *Biochem. J.* **204**, 103 (1982).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
15. H. L. Bonkowsky and J. S. Pomeroy, *Clin. Sci. molec. Med.* **52**, 509 (1977).
16. B. Grandchamp, J. C. Deybach, M. Grelier, H. de Verneuil and Y. Nordmann, *Biochim. biophys. Acta* **629**, 577 (1980).
17. K. E. Anderson, A. P. Alvares, S. Sassa and A. Kappas, *Clin. Pharmac. Ther.* **19**, 47 (1976).
18. B. B. Brodie, J. Axelrod, R. Soberman and B. B. Levy, *J. biol. Chem.* **179**, 25 (1949).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. J. E. Falk, *Porphyrins and Metalloporphyrins*, p. 236. Elsevier, Amsterdam (1964).
21. M. Barry and S. Sherlock, *Lancet* **i**, 100 (1971).
22. P. Raj, E. R. Nelson and R. W. Estabrook, *Chem. Biol. Interact.* **3**, 303 (1971).
23. J. F. Sinclair, P. R. Sinclair and H. L. Bonkowsky, *Biochem. biophys. Res. Commun.* **86**, 710 (1979).
24. P. Sinclair, J. Bement, J. Sinclair, H. Bonkowsky, G. Dannan and S. Aust, *Toxicologist* **1**, 71 (1981).
25. D. Mauzerall, *J. Am. chem. Soc.* **84**, 2437 (1962).

Biochemical Pharmacology, Vol. 33, No. 3, pp. 502–505, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Preventive effect of cysteine on butylated hydroxytoluene-induced pulmonary toxicity in mice

(*Received* 16 *May* 1983; *accepted* 4 *August* 1983)

Butylated hydroxytoluene (BHT)* is widely used as an antioxidant in processed foods and petroleum products. Though generally considered to be safe at the concentration present in foods (the acceptable daily intake of BHT for man is 0.5 mg/kg [1]), high doses of this compound cause pulmonary hypertrophy, hyperplasia and general disorganization of the cellular components in mice [2–5]. The mechanism by which BHT causes such damage has not yet been elucidated, but it has been presumed that such damage is due to the interaction between reactive metabolites and some cellular components.

We have been studying the effect of this compound on cellular macromolecules [6–11]. Previous studies have demonstrated that (a) BHT is converted to highly reactive intermediates by a cytochrome P-450-linked monooxygenase system in the microsomes [6, 7], and (b) some of the activated metabolites, BHT-quinone methide and BHT-alcohol, specifically bind to a sulfhydryl group of cellular protein [9, 11]. In an *in vitro* study [7, 11], we found that cysteine and reduced glutathione significantly decreased the binding of BHT to cellular protein.

Based on previous *in vivo* and *in vitro* results, we investigated whether or not dietary cysteine could prevent pulmonary enlargement induced by BHT *in vivo*.

Methods and Materials

Animals. Four-week-old male mice of the BALB/cAn strain were obtained from Charles River Japan Inc. (Atsugi, Japan). The animals were housed in plastic cages on hard-wood laboratory bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY). Since Malkinson [12] reported that BHT-produced pulmonary damage was prevented by cedar terpenes derived from shavings used for cage bedding, the hard-wood laboratory bedding was employed in all experiments.

Chemicals. BHT (toluene[*methyl*-^{14}C]) (specific radioactivity, 0.485 μCi/μmole) was purchased from the New England Nuclear Corp. (Boston, MA). The radiochemical purity of the compound was rechecked by thin-layer chromatography and found to be more than 99%. Non-radioactive BHT was obtained from the Wako Pure Chemicals Co. (Osaka, Japan), and all other chemicals used were of the highest obtainable purity.

Experimental protocol. In the initial experiments, mice were fed a commercial stock diet (CLEA CE-2, CLEA Japan Inc., Tokyo) for 3 weeks. They were then fed a diet supplemented with L-cysteine or another L-amino acid, which was added to the CE-2 diet, for 3 days. After the 3 days, each mouse was given a solution of BHT dissolved in corn oil (0.1 ml/20 g body wt) by intraperitoneal (i.p.) injection each day for 4 days. During this time they were maintained on the supplemented diet. The corresponding control animals received an equivalent volume of corn oil. Twenty-four hours after the last BHT dose, mice were decapitated under anesthesia with ether, and the lungs were removed and weighed. Trapped blood was not apparent in the tissues of the decapitated animals. Measurement of lung weight was used in most experiments to monitor whether pulmonary damage had occurred [3, 12].

In a second series of experiments, after feeding a commercial stock diet for 3 weeks, mice were fed a 1% cysteine-supplemented diet for 3 days. Then the animals received 200 mg/kg of BHT containing [^{14}C]BHT dissolved in corn oil (0.1 ml/20 g body wt, approximately 1.5 to 1.6 μCi/animal). Mice were decapitated 6 hr after i.p. injection of [^{14}C]BHT. The lungs and liver were perfused with saline and homogenized with 1.15% KCl in a Polytron homogenizer (Kinematica GmbH, Switzerland). Radioactivity covalently bound to tissue macromolecules was determined by the procedure described previously [6, 8].

Other methods. Radioactivity was measured by a Beckman scintillation spectrometer, model LS-355, and compared with external standard. The scintillation medium used consisted of 2 vol. toluene phospher (4 g PPO and 100 mg dimethyl-POPOP per 1000 ml of toluene) and 1 vol. Triton X-100 [13]. Protein content was measured by the biuret method [14].

Results and Discussion

Figure 1 shows the effect of dietary cysteine on pulmonary enlargement induced by BHT in mice. The relative

* Abbreviations: BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; BHT-alcohol, 2,6-di-*tert*-butyl-4-hydroxymethylphenol; BHT-quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.