Biochemical Pharmacology, Vol. 34, No. 8, pp. 1133–1136, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

DIFFERENT EFFECTS OF CYANIDE ON THE ACTIVITIES OF 7-ETHOXYCOUMARIN O-DEETHYLATION CATALYZED BY TWO FORMS OF CYTOCHROME P-450 PURIFIED FROM 3-METHYLCHOLANTHRENE-TREATED RATS

MITSUKAZU KITADA*, KEN'ICHI SAKAMOTO, TADAAKI RIKIHISA and YOSHIO KANAKUBO
Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba 260, Japan

(*Received* 19 *June* 1984; *accepted* 6 *September* 1984)

Abstract—The effect of cyanide on 7-ethoxycoumarin O-deethylation by two cytochrome P-450 isozymes obtained from 3-methylcholanthrene treated rat liver microsomes was investigated. 7-Ethoxycoumarin O-deethylation was stimulated by the addition of cyanide to a reconstituted monooxygenase system consisting of NADPH, dilauroyl 3-L-phosphatidylcholine, NADPH-cytochrome P-450 reductase and MC P-448$_2$ (low spin form of cytochrome). In contrast, a weak inhibitory effect of cyanide on 7-ethoxycoumarin O-deethylation was observed when MC P-448$_1$ (high spin form of cytochrome) was used in the reconstituted system. Cyanide did not influence the apparent K_m for 7-ethoxycoumarin when either form of cytochrome P-450 was used in the reconstituted system and did not stimulate the cumene hydroperoxide dependent O-deethylation by MC P-448$_2$. The stimulatory effect of cyanide on O-deethylation by MC P-448$_2$ was decreased with increasing the concentration of the reductase added to the reconstituted system. On the other hand, the effect of cyanide on O-deehtylation by MC P-448$_1$ was virtually independent on the amount of the reductase added.

In recent years, cytochrome P-450 has been established to be a family of isozymes with differing substrate specificities and spectral properties [1]. The isozymes are induced by pretreatment of rats with drugs and chemical compounds such as 3-methylcholanthrene, 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD), phenobarbital and polychlorinated biphenyl. The 3-methylcholanthrene or TCDD-inducible forms of cytochrome P-450 (often termed cytochrome P-448) differs markedly in their spectral and catalytic properties from those of control or phenobarbital induced rats [2, 3]. In addition, the existence of at least two cytochrome P-448 with differing substrate specificities, molecular weight and spectral properties have been demonstrated by several investigators [4–7]. More recently, a form of cytochrome P-450 which is clearly different from the other major form induced by 3-methylcholanthrene has been isolated from rats treated with this compound [8]. In the present study, we describe the effect of cyanide on the activities of 7-ethoxycoumarin O-deethylation catalyzed by two forms of cytochrome P-450 purified from 3-methylcholanthrene treated rats.

MATERIALS AND METHODS

Preparation of microsomal enzymes. Liver microsomes from phenobarbital treated (0.1% sodium phenobarbital added to drinking water for 7 days) and 3-methylcholanthrene treated (25 mg/kg, intraperitoneally on the 2nd, 4th and 8th day before sacrifice)-male Sprague–Dawley rats weighing 120–160 g were prepared by a standard procedure [9]. Cytochromes P-450 (which will be called MC P-448$_1$ and MC P-448$_2$ in the present study) were purified from 3-methylcholanthrene treated rats by a minor modification of the methods previously described [10–14]. Polyacrylamide gel electrophoresis by the method of Laemmli [15] indicated a single major band in each. The final preparations used had specific contents of 13.9 nmole MC P-448$_1$ per mg of protein and 15.5 nmole MC P-448$_2$ per mg of protein, respectively. MC P-448$_1$ was high spin in the native state with characteristic absorption peaks at 391 and 644 nm. MC P-448$_2$ was low spin with a Soret peak at 417 nm. The ferrous cytochrome-carbon monoxide complex of both cytochromes had maxima at 447 nm. The molecular weight of the two cytochromes were approximately 53,000 and 55,000 for MC P-448$_1$ and MC P-448$_2$, respectively. NADPH-cytochrome P-450 reductase was solubilized with Emulgen 913 from liver microsomes of phenobarbital treated rats and purified by the method of Yasukochi and Masters [16] with minor modification. The final preparation had a specific activity of 47.1 unit per mg of protein.

Incubation condition. The incubation mixture consisting of 100 mM potassium phosphate (pH 7.25), 25 μg dilauroyl 3-L-phosphatidylcholine, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit glucose 6-phosphate dehydrogenase and 6 mM $MgCl_2$), 7-ethoxycoumarin, cytochrome P-450 and the reductase (refer to figures and tables for quantities) in a final volume of 0.5 ml. When the effect of oxygen tension on the reaction was studied, incubation was con-

* To whom all correspondence should be addressed.

ducted under the gas phase containing a various concentration of oxygen ($O_2 : N_2 = 20 : 80, 50 : 50$ or $80 : 20$). The reaction was started by the addition of NADPH-generating system.

Assay methods. Cytochrome P-450 was determined according to the method of Omura and Sato [17]. NADPH-cytochrome *c* (P-450) reductase activity was measured by the method of Phillips and Langdon [18]. The concentration of the reductase was determined from the absorbance at 456 nm in the absolute spectrum using an extinction coefficient of 21.4 mM^{-1} cm^{-1} [19]. Protein was assayed by the method of Lowry *et al.* [20] using bovine serum albumin as a standard. 7-Ethoxycoumarin O-deethylation activity was measured by determining 7-hydroxycoumarin by the method of Aitio [21].

Reagents and biochemicals. NADP, NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Osaka, Japan and cytochrome *c* (horse heart) was from Sigma. benzphetamine and Emulgen 913 were kindly provided by the Upjohn Co. (Kalamazoo, MI), and Kao Atlas Co. (Japan), respectively. All other chemicals were of the highest purity commercially available.

RESULTS AND DISCUSSION

The effect of cyanide on 7-ethoxycoumarin O-deethylation by a reconstituted system in which reductase is present in limiting amount is shown in Fig. 1. The addition of cyanide to the reconstituted system containing MC P-448$_2$ resulted in an increase in 7-ethoxycoumarin O-deethylation. The stimulation of MC P-448$_2$-dependent reaction was observed between the concentration of 0.25 mM and 1.0 mM cyanide, and was gradually decreased with increasing the concentration of cyanide employed. On the contrary, the MC P-448$_1$-dependent reaction was slightly inhibited by the addition of cyanide. Such a difference in the effect on cytochromes P-450 indicates that the action of cyanide may be due to interaction with cytochrome P-450 rather than

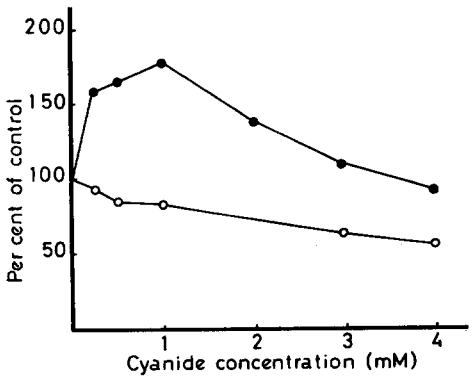

Fig. 1. Effect of potassium cyanide on 7-ethoxycoumarin O-deethylation catalyzed by MC P-448$_1$ or MC P-448$_2$. The concentrations of MC P-448$_1$, MC P-448$_2$ and reductase used were 0.08, 0.07 and 0.14 μM, respectively. The activities of 7-ethoxycoumarin O-deethylation in the absence of cyanide were 15.6 (MC P-448$_1$; ○) and 15.6 (MC P-448$_2$; ●) nmole product/min/nmole cytochrome P-450, respectively.

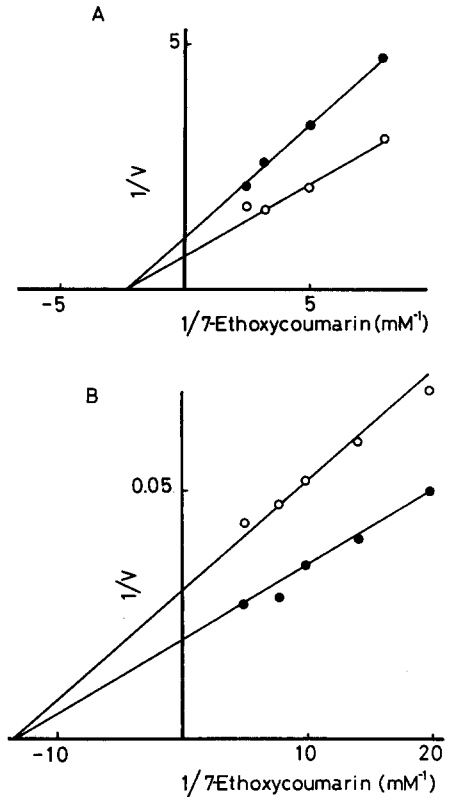

Fig. 2. Double reciprocal plot of 7-ethoxycoumarin O-deethylation activity and 7-ethoxycoumarin concentration. The concentrations of MC P-448$_1$ (A), MC P-448$_2$ (B) and reductase were the same as described in Fig. 1. MC P-448$_1$-dependent O-deethylation activity was measured in the presence (●) or absence (○) of 4 mM potassium cyanide, and MC P-448$_2$-dependent reaction was conducted in the presence (●) or absence (○) of 1 mM potassium cyanide.

reductase. Figure 2 shows the effect of cyanide on the Michaelis constant (K_m) and maximal velocity of 7-ethoxycoumarin O-deethylation catalyzed by MC P-448$_1$ or MC P-448$_2$. It can be seen that the addition of cyanide did not cause any change in an apparent K_m of 7-ethoxycoumarin during O-deethylation by either MC P-448$_1$ or MC P-448$_2$ (MC P-448$_1$; 0.4 mM, MC P-448$_2$; 0.07 mM). In addition, the activities of cumene hydroperoxide-dependent O-deethylation reaction catalyzed by MC P-448$_2$ in the presence and absence of 1 mM cyanide were 5.29 ± 0.21 and 4.67 ± 0.05 nmole/min/nmole cytochrome P-450, respectively (mean ± S.D. N = 3). The lack of effect of cyanide on K_m for 7-ethoxycoumarin and the lack of the stimulatory effect of cyanide on cumene hydroperoxide-dependent reaction by MC P-448$_2$ indicate that cyanide does not affect the affinity of the substrate for either cytochromes P-450 and does not enhance cumene hydroperoxide-mediated formation of oxidized MC P-448$_2$ peroxide anion or subsequent steps leading to 7-hydroxycoumarin. Cyanide interacted with MC P-448$_2$ to produce absorption spectra characterized by a trough at 410 nm and a peak at 442 nm. From double reciprocal plot of the spectral change induced by cyanide and cyanide concentration, spectral dissociation constant

(K_s) of cyanide for MC P-448$_2$ was found to be 8 mM. Type II compounds are generally considered to interact with the sixth ligand of the cytochrome [21] and to inhibit microsomal drug oxidations [9, 22]. However, it has been shown that cyanide exerted a stimulatory effect as well as an inhibitory effect on aniline hydroxylation in rat liver microsomes, and that the extents of these two effects were differently affected by reaction conditions such as oxygen tension [23]. Therefore, the effect of oxygen tension on the reaction in the presence and absence of cyanide was studied. As shown in Table 1, cyanide induced enhancement of MC P-448$_2$-dependent reaction was increased with increasing oxygen tension suggesting that cyanide may compete with molecular oxygen for reduced form of the cytochrome and may inhibit the reaction. In other words, these results suggest that cyanide exerts not only a stimulatory effect but also an inhibitory effect on the MC P-448$_2$-dependent reaction. Therefore, it seems possible to assume that a decrease in the stimulatory effect of cyanide on MC P-448$_2$-dependent reaction at higher concentration of cyanide may be due to the inhibitory effect of cyanide.

Effect of reductase concentration on the cyanide effect of 7-ethoxycoumarin O-deethylation is shown in Fig. 3. As can be seen in Fig. 3, the effect of cyanide on MC P-448$_1$-dependent reaction was essentially independent of the concentration of reductase added to the reconstituted system. On the contrary, the stimulatory effect of cyanide on MC P-448$_2$-dependent reaction was decreased as the concentration of reductase was increased (Fig. 3B). Furthermore, the apparent K_m of MC P-448$_2$ for reductase in the absence of cyanide was much higher than that in the presence of 1 mM cyanide (0.09 vs 0.29 μM). The effects of oxygen tension and reductase concentration on the stimulatory effect of cyanide observed in the present study were in accord with previous results [23], but the effect of substrate concentration on the cyanide effect was not. Although inhibition of aniline hydroxylation by cyanide was observed when aniline concentration was lower than about 3 mM, and activation was observed when aniline concentration was higher than 3 mM [23], the effect of cyanide on 7-ethoxycoumarin O-deethylation was independent upon substrate concentration. Furthermore, in this study, we used cytochromes P-450 purified from 3-methylcholanthrene

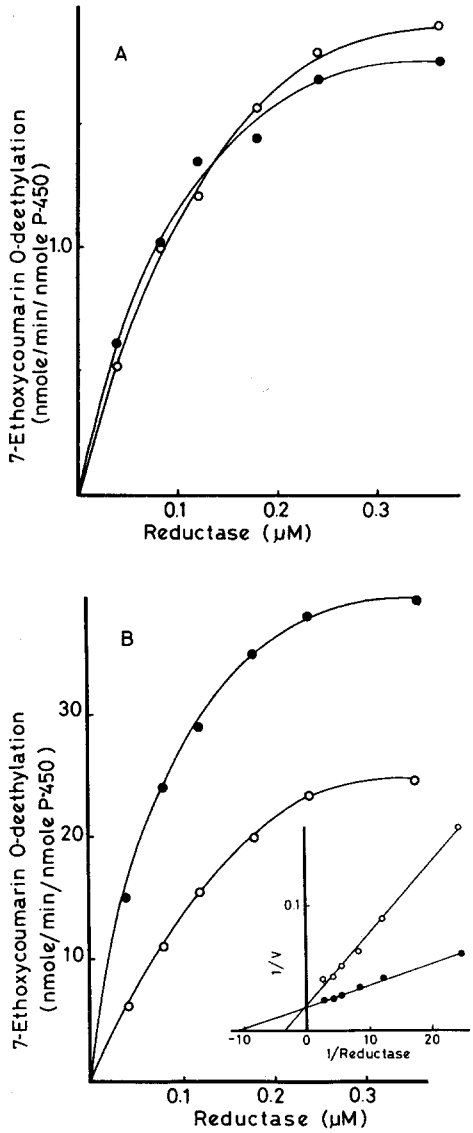

Fig. 3. Effect of potassium cyanide on 7-ethoxycoumarin O-deethylation in the presence of various concentrations of reductase. The concentration of MC P-448$_1$ (A) or MC P-448$_2$ (B) was 0.09 μM. O-Deethylation activity was determined in the presence (●) or absence (○) of 1 mM potassium cyanide.

Table 1. Effect of oxygen tension on 7-ethoxycoumarin O-deethylation in the presence or absence of potassium cyanide

Oxygen tension (%)	7-Ethoxycoumarin O-deethylation (nmole product/min/nmole cyt.) −KCN	+KCN (2 mM)	Enhancement (%)
20	16.6	20.7	25.5
50	15.1	22.9	51.9
80	15.8	30.3	91.8

The concentrations of MC P-448$_2$ and reductase used were 0.06 and 0.12 μM, respectively.

treated rats, on the other hand, as reported previously [23] 3-methylcholanthrene treatment resulted in the decrease in the enhancing effect on aniline hydroxylation in liver microsomes. Therefore, the possibility that the mechanism of cyanide-induced stimulation observed in this study is different from that of cyanide-induced enhancement of aniline hydroxylation in liver microsomes cannot be excluded.

Several chemicals such as ethylisocyanide [24], acetone [25], 7,8-benzoflavone [26] and metyrapone [27] are known to enhance drug oxidation reactions. Recently, Huang *et al.* [28] have shown that 7,8-benzoflavone decreases the apparent K_m for reductase and increases V_{max} for benzo(*a*)pyrene

hydroxylation in human, rabbit and hamster liver microsomes. The results in the present study suggest that the increase in the affinity of cytochrome P-450 for reductase may in part be responsible for the enhancement of MC P-448$_2$-mediated 7-ethoxycoumarin O-deethylation. Further work is, however, needed to conclude a definite mechanism of difference in the response of cytochrome P-450 to cyanide and that of cyanide enhancement.

REFERENCES

1. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1980).
2. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979)
3. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **256**, 1044 (1981).
4. J. A. Goldstein, P. Linko, M. I. Luster and D. W. Sunderheimer, *J. biol. Chem.* **257**, 3950 (1982).
5. T. Kamataki, K. Maeda, Y. Yamazoe, N. Matsuda, K. Ishii and R. Kato, *Molec. Pharmac.* **24**, 146 (1983).
6. D. W. Sunderheimer, N. B. Caveness and J. A. Goldstein, *Arch. Biochem. Biophys.* **226**, 548 (1983).
7. L. M. Reik, W. Levin, D. E. Ryan and P. E. Thomas, *J. biol. Chem.* **257**, 3950 (1982).
8. C. R. Wolf and F. Oesch, *Biochem. biophys. Res. Commun.* **111**, 504 (1983).
9. M. Kitada, K. Chiba, T. Kamataki and H. Kitagawa, *Japan. J. Pharmac.* **27**, 601 (1977).
10. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **8**, 8 (1974).
11. C. Hashimoto and Y. Imai, *Biochem. biophys. Res. Commun.* **68**, 821 (1976).
12. M. Warner, M. V. La Marca and A. H. Neims, *Drug Metabol. Dispos.* **6**, 353 (1978).
13. T. Kamataki, K. Maeda, Y. Yamazoe, T. Nagai and R. Kato, *Biochem. biophys. Res. Commun.* **103**, 1 (1981).
14. F. P. Guengerich, G. A Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
15. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
16. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
17. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
18. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
19. J. S. French, F. P. Guengerich and M. J. Coon, *J. biol. Chem.* **255**, 4112 (1980).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. J. B. Shenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmac.* **3**, 113 (1967).
22. T. Matsubara and Y. Tochino, *Arch. biochem. Biophys.* **172**, 696 (1976).
23. T. Kamataki, M. Kitada, K. Chiba, H. Kitagawa, Y. Imai and R. Sato, *Biochem. Pharmac.* **29**, 1141 (1980).
24. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **25**, 80 (1966).
25. M. W. Anders, *Archs. Biochem. Biophys.* **126**, 269 (1968).
26. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
27. K. C. Leibman, *Molec. Pharmac.* **5**, 1 (1969).
28. M.-T. Huang, R. L. Chang, J. G. Fortner and A. H. Conney, *J. biol. Chem.* **256**, 6829 (1981).