

METABOLISM AND DISPOSITION OF CHLORZOXAZONE IN RATS: EFFECTS OF ETHANOL AND DISULFIRAM

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ABSTRACT: *Role of rat cytochrome P-450 2E1 (P-450 2E1) in the metabolism of chlorzoxazone was examined by using several approaches; (1) selective inhibition of catalytic activity in rat liver microsomes by diethyldithiocarbamate, (2) correlation of dimethylnitrosamine N-demethylation with chlorzoxazone 6-hydroxylation, and (3) immunoinhibition of catalytic activity with rabbit anti-rat P-450. The results indicated that P-450 2E1 is responsible for the metabolism of chlorzoxazone. In order to examine the role of P-450 2E1 activity on the in vivo disposition of chlorzoxazone, plasma concentration of chlorzoxazone and urinary excretion of 6-hydroxychlorzoxazone were determined in rats pretreated either with ethanol (P-450 2E1 inducer) or with disulfiram (P-450 2E1 inhibitor). Plasma clearance and urinary excretion were significantly enhanced in ethanol treated group while inhibited in disulfiram treated group. These results suggest that chlorzoxazone is metabolized selectively by P-450 2E1 in vivo and that drug metabolizing capacity is important in the disposition of drugs.*

Key Words: *Chlorzoxazone, ethanol, disulfiram, cytochrome P-450*

INTRODUCTION

Chlorzoxazone (5-chloro-2(3H)-benzoxazolone) has been widely used as a muscle relaxant for spasm since it was first introduced in 1958 (Axoury, 1982). Chlorzoxazone is mainly metabolized by cytochrome P-450 enzyme in liver and excreted into urine as a glucuronide conjugate (Conney and Burns, 1960). Recently human CYP 2E1 has been reported to play a major role in chlorzoxazone metabo-

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lism (Guengerich *et al.*, 1991). Cytochrome P-450 enzyme exists in multiple forms and is involved in the oxidative metabolism of drugs, carcinogens, and natural products as well as endogenous compounds such as steroids, fatty acids, and eicosanoids (Guengerich, 1987). Cytochrome P-450 2E1 is mainly involved in the metabolism of low molecular weight toxicants and the activity can be induced by ethanol and other organic solvents (Yoo *et al.*, 1987; Yang *et al.*, 1990). Interindividual variance in the level of P-450 2E1 is significant due to the drinking and some food ingredients. This variance can influence the toxicological behavior of chemicals metabolized by this enzyme.

The purpose of this study was to determine the role of rat liver P-450 2E1 in the metabolism of chlorzoxazone and to examine the effects of ethanol and disulfiram, modulators of P-450 2E1 activity, on the disposition of chlorzoxazone in rats.

MATERIALS AND METHODS

Reagents

Chlorzoxazone, 7-hydroxycoumarine, pyridine, and acetic anhydride were purchased from Aldrich Chemical Co. (Milwaukee, IL, USA). Heparine, acetaminophen, 3-methylcholanthrene, and β -glucuronidase were obtained from Sigma Co. (St. Louis, MO, USA). MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) was purchased from Mecherey & Nagel (Germany). 6-Hydroxy chlorzoxazone and 5-fluoro-2-benzoxazolone were kindly donated from Dr. F.P. Guengerich (Vanderbilt Univ. USA). All other chemicals used were highest grade commercially available.

Animals

Male Sprague-Dawley rats (200-250 g) were used in all experiments. All animals were maintained on Lab Chow (Samyang, Korea) and tap water *ad libitum*.

In vitro assays

Animals were treated with various compounds at the following dosage: phenobarbital (PB), 80 mg/kg in 0.15 M NaCl administered ip once each day for 3 days; 3-methylcholanthrene (3-MC), 25 mg/kg in corn oil ip once each day for 3 days; acetone, 5 ml/kg po 24 hr before sacrifice. Animals were killed 24 hr after last dosing. Livers were removed and microsomes were prepared as described elsewhere (Guengerich, 1989).

Chlorzoxazone hydroxylation was carried out as described elsewhere (Peter *et al.*, 1990), and the products were monitored by a HPLC system using 5-fluoro-2-benzoxazolone as an internal standard. Enzymatic dimethylnitrosamine N-demethylation was assayed colorimetrically according to Tu *et al.* (1983) using NADPH-generating system to support the reaction.

Inhibition Studies

Immunoinhibition experiments were carried out by preincubating 50 pmol of total P-450 in microsomes in 375 μ l of 0.1 M potassium phosphate buffer (pH 7.4) with various amounts of antibodies (IgG) or preimmune IgG fraction as control

for 30 min at 25°C. Rabbit anti rat P-450 antibodies were obtained from Dr. F.P. Guengerich (Vanderbilt Univ.). The assay was started with NADPH-generating system after the addition of substrate and additional incubation for 20 min at 37°C. Inhibition by diethyldithiocarbamate was tested by preincubation of inhibitor and microsomes for 3 min at 37°C.

Administration of chlorzoxazone into rats

Rats in groups of 3 were pretreated as following; 1 ml physiological saline po once a day for 3 day; ethanol, 5 ml/kg po once 24 hr before experiment; disulfiram, 50 mg/kg po once a day for 3 days. Animals were anesthetized with sodium pentobarbital (50 mg/kg) and femoral vein was cannulated with polyethylene tube (PE-50). Then chlorzoxazone was orally administered at a dose of 50 mg/kg. Blood samples (0.25 ml) were drawn through femoral vein at 0 (predose), 0.33, 0.66, 1, 1.5, 2, 4 and 6 hr after dosing. The samples were then centrifuged to obtain plasma and stored at 4°C until analyzed. Urine samples were collected from separate groups for the following periods: 0 (predose), 0-2, 2-4, 4-6, 6-8, 8-24 and 24-36 hr after dosing.

Analysis of plasma samples for chlorzoxazone

0.1 ml of plasma was added to 0.4 ml of phosphate buffered saline (pH 7.0) and 10 μ l of acetaminophen (300 ppm) as an internal standard. The samples were then extracted with 1 ml of ethylacetate. The organic layer was taken and evaporated to dryness. To the residue were added 5 μ l of pyridine and 15 μ l of acetic anhydride. The tubes were capped and placed in a water bath at 42°C for 30 min. After the acetylation, the samples were dried and redissolved in 50 μ l of ethylacetate. The analysis of chlorzoxazone was done by gas chromatography (HP 5890) equipped with NPD.

Analysis of Urinary 6-Hydroxychlorzoxazone

0.5 ml of urine samples was mixed with 1 ml potassium phosphate buffer (0.1 M, pH 7.0) and 50 μ l of 7-hydroxycoumarine (1000 ppm) as an internal standard. In order to hydrolyze glucuronide conjugates, 100 unit of β -glucuronidase (from *E. coli*) was added to the samples and the mixtures were incubated at 50°C for 1 hr. The samples were then extracted twice with 2 ml ethylacetate and combined ethylacetate solution was completely dried under nitrogen gas. The residue was then silylated by the incubation with 30 μ l of MSTFA and 70 μ l of acetonitrile at 80°C for 10 min. An 1 μ l of aliquot of the resulting reaction mixture was injected into gas chromatography equipped with FID.

Statistical Analysis

Pharmacokinetic analysis was done using a nonlinear regression program, PC-NONLIN (Statistical Consultants Inc., Lexington, KY. USA).

RESULTS AND DISCUSSION

***In Vitro* Metabolism of Chlorzoxazone**

Cytochrome P-450 plays a critical role in the oxidative metabolism of xenobio-

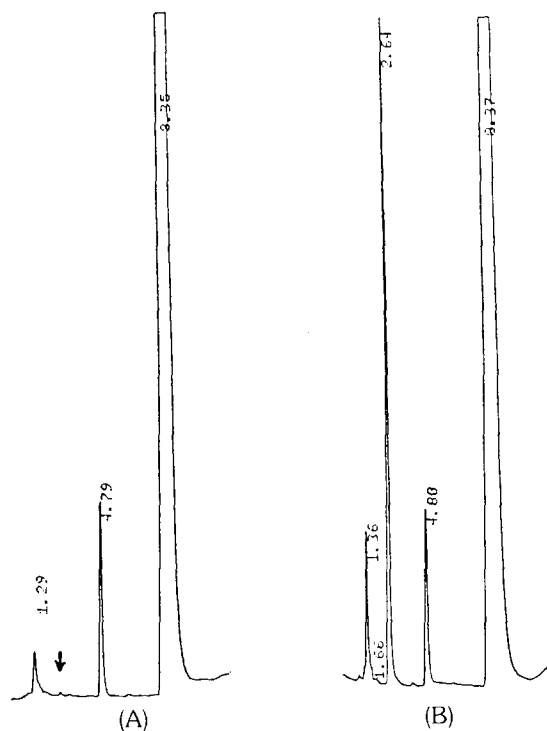


Figure 1. HPLC chromatograms of incubations assayed for chlorzoxazone 6-hydroxylation. Rat liver microsomes were incubated with 0.5 mM chlorzoxazone for 20 min. (A) microsomes incubated without an NADPH-generating system (arrow indicates retention time of 6-hydroxy chlorzoxazone); (B) microsomes plus an NADPH-generating system. The retention times of 6-hydroxychlorzoxazone, 5-fluoro-2(3)H-benzoxazolone (internal standard), and chlorzoxazone are 2.64, 4.80 and 8.37 min, respectively.

tics such as drugs, toxicants, and other natural products as well as endogenous compounds (Guengerich, 1987 and references therein). This enzyme is multigene family and more than 10 subtypes have been characterized in rats (Gonzalez, 1989). P-450 enzymes generally show broad substrate specificity and can be induced by many compounds such as ethanol, phenobarbital and cigarette smoking. The induction or inhibition of this enzymes along with genetic polymorphism attributes to the interindividual differences in drug metabolism and gives a profound effects on the drug clearance.

In vitro incubation of chlorzoxazone with rat liver microsome produced only one metabolite 6-OH chlorzoxazone (Figure 1). Dehalogenated benzoxazolone, known to be minor metabolite (Twele and Spiteller, 1982), has not been detected in our analytical system. In order to demonstrate the role of rat P-450 2E1 in the oxidation of chlorzoxazone, correlation experiment, selective inhibition by diethyldithiocarbamate, and immunoinhibition approach were used.

Different microsomes prepared from control, PB-, 3-MC- and acetone-treated groups were applied to examine the relationship between dimethylnitrosamine-N-demethylation and chlorzoxazone-6-hydroxylation. A significant relationship was noted ($r=0.867$) between the metabolism of chlorzoxazone and that of dimethyl-

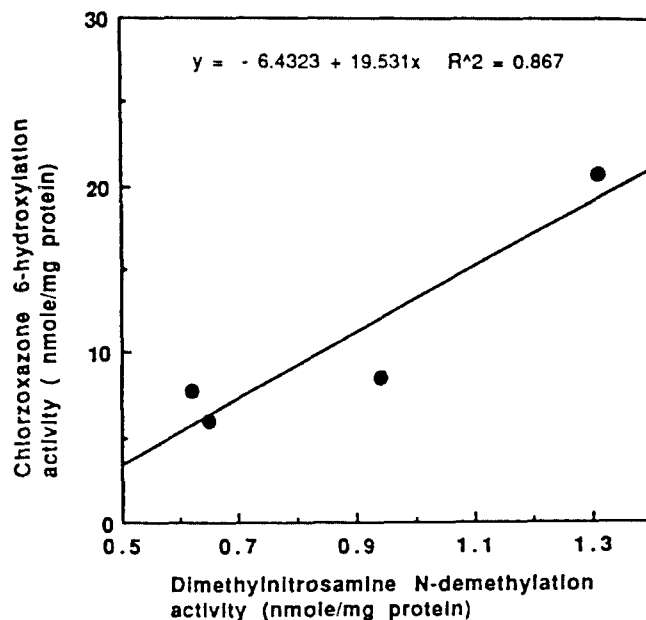


Figure 2. Correlation of catalytic activity between rates of chlorzoxazone 6-hydroxylation and dimethylnitrosamine N-demethylation in different rat liver microsomes. Untreated, phenobarbital, 3-methylcholanthrene, and acetone-treated microsomes were used in this experiments. Line was drawn by linear regression analysis, and correlation coefficients (r) was calculated.

nitrosamine, suggesting that both compounds are metabolized by the same P-450 (Figure 2). Several lines of evidence indicated that dimethylnitrosamine was metabolized specifically by P-450 2E1 at low concentrations (Koop *et al.*, 1989; Yoo *et al.*, 1987). Diethyldithiocarbamate and disulfiram, its oxidized form, were first reported to be mechanism-based inactivators by Hunter and Neal (1975) and later these compounds have been found to be rather selective for P-450 2E1 (Guenge-*rich et al.*, 1991) although the exact nature of this inactivation process has not been characterized. Varying concentrations (10 to 300 μM of diethyldithiocarbamate considerably inhibited chlorzoxazone 6-hydroxylation activity in rat liver microsomes (Figure 3). Of antibodies tested, only anti P-450 2E1 inhibited the formation of 6-hydroxychlorzoxazone while anti P-450 2B1, anti P-450 2D1, and anti P-450 1A2 had no inhibitory effects (Table 1). The inhibition of chlorzoxazone 6-hydroxylation was dependent on the amounts of immunoglobulin G protein. Metabolite formation of chlorzoxazone was reduced by 88% at concentration of 4.5 mg of immunoglobulin G protein/nmol of P-450. By the above three approach, evidence was obtained that in rat liver P-450 2E1 is a major, if not principal, catalyst of the metabolism of chlorzoxazone.

In Vivo Disposition of Chlorzoxazone: Effects of Ethanol and Disulfiram

Several studies demonstrated that chlorzoxazone is eliminated almost entirely by the metabolism and only a small portion is eliminated as parent form (Conney

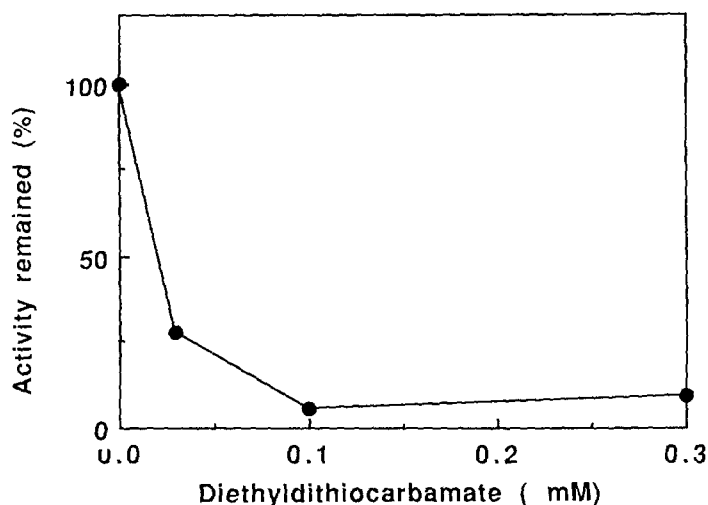


Figure 3. Inhibition of chlorzoxazone 6-hydroxylation in rat liver microsomes by diethyldithiocarbamate. chlorzoxazone and inhibitor were added to the microsomes and the reaction was initiated by the addition of an NADPH-generating system.

Table 1. Immunoinhibition of catalytic activities in rat liver microsomes.

Antibody	%of uninhibited chlorzoxazone-6-hydroxylation activity
preimmune,	
5 mg/nmol of P-450	95
anti P-450 2B1,	
2 mg/nmol of P-450	103
anti P-450 2D1,	
5 mg/nmol of P-450	89
anti P-450 1A2,	
5 mg/nmol of P-450	121
anti P-450 2E1	
0.4 mg/nmol of P-450	72
1.5 mg/nmol of P-450	43
4.5 mg/nmol of P-450	12
10 mg/nmol of P-450	2

and Burns, 1960; Desiraju *et al.*, 1983). Thus changes in the clearance of chlorzoxazone from the body reflect changes in overall rates of metabolism.

The mean plasma chlorzoxazone concentration-time profile is shown in Figure 4. Chlorzoxazone was rapidly absorbed, attaining a mean peak level of $31.66 \pm 5.05 \mu\text{g/ml}$. Relatively high interindividual differences were observed in the plasma level. This variance is probably due to the metabolic differences among them. After reaching the peak level, the concentration of chlorzoxazone was decreased in a monoexponential manner. Urinary analysis demonstrated that chlorzoxazone was excreted into urine as a glucuronide conjugate and only a trace amount of chlorzoxazone was detected. Our results is consistent with previous data reported by Con-

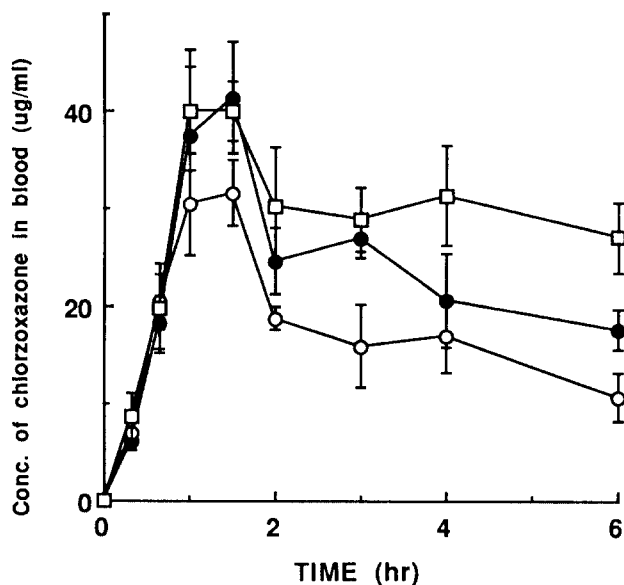


Figure 4. Mean (\pm SD) plasma concentrations of chlorzoxazone (ug/ml) in rats following oral administration of chlorzoxazone (50 mg/kg). Rats were pretreated with ethanol (-○-) or disulfiram (-□-) or nothing (-●-) before chlorzoxazone.

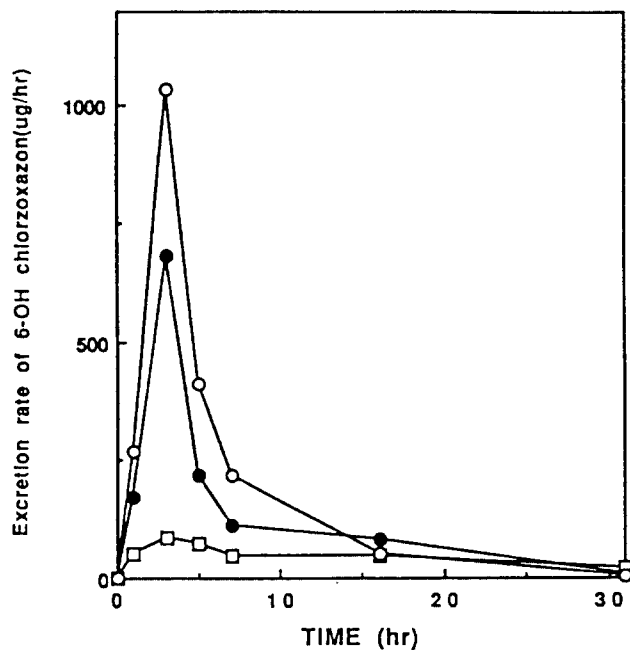


Figure 5. Urinary excretion of 6-hydroxychlorzoxazone in rats following administration of chlorzoxazone (50 mg/kg); no treatment (-●-), ethanol treated group (-○-), and disulfiram treated group (-□-).

Table 2. Pharmacokinetic parameters following oral dose (50 mg/kg) of chlorzoxazone in rats pretreated either with ethanol or with disulfiram.

Parameter	Control	Ethanol	Disulfiram
Ka (hr ⁻¹)	1.135	1.132	1.285
K (hr ⁻¹)	0.214	0.317	0.094
t _{1/2} (hr)	3.23	2.19	7.94
AUC ^{0-t} (μg*hr/ml)	141	103	173
AUC ^{0-∞} (μg*hr/ml)	213	130	447
MRT (hr)	5.54	4.04	11.44
Vd/f (ml/kg)	1091	1212	1192

all values are mean of three in each group.

ney and Burns (1960). Urinary excretion showed the maximum rate of 683 ± 54 μg/hr at 3 hr after dosing and more than 80% of total urinary 6-hydroxychlorzoxazone was excreted within first 8 hr (Figure 4). These results suggest that chlorzoxazone is decreased in a simple exponential manner and metabolized to 6-hydroxychlorzoxazone before excreted into urine. Therefore hepatic metabolism seems to be a rate-limiting step in chlorzoxazone body clearance.

In order to examine the effects of modulation of *in vivo* P-450 activity on the chlorzoxazone disposition, plasma concentration and urinary excretion were measured either in ethanol pretreated group or in disulfiram pretreated group. In case human, the level of P-450 2E1 differs dramatically among humans and may be regulated by several agents and disease states (Yang *et al.*, 1990). Thus the toxicity of chemicals that are metabolically activated by P-450 2E1 may depend entirely on the level of P-450 2E1. Disulfiram has been known to be a potent P-450 inhibitor *in vivo* and diethyldithiocarbamate, its reduced form, was demonstrated strong inhibitor of P-450 2E1 (Kim and Guengerich, 1990; Guengerich *et al.*, 1991). Ethanol has been reported to be a strong P-450 2E1 inducer (Yoo *et al.*, 1987; Koop *et al.*, 1989). As shown in Figure 4 and Figure 5, the plasma concentration of chlorzoxazone and urinary excretion of 6-hydroxychlorzoxazone in either group were significantly changed when compared to control group. Changes in absorption rate constant (*ka*) were not observed among groups while apparent elimination (*k*) constant and AUC were differed significantly (Table 2). Total urinary excretion of 6-hydroxychlorzoxazone in control, ethanol-treated, and disulfiram-treated groups was 37.6 ± 4.1 , 46.2 ± 5.7 and $14.6 \pm 2.5\%$, respectively. These data suggest that absorption of chlorzoxazone was not affected by the treatment of ethanol or disulfiram and body elimination was totally dependent on the metabolism. It is difficult to maintain the effective blood concentration of drugs in extensive metabolizer. In case of low metabolic capacity, the toxic effects might appear due to high blood concentration or metabolic shunt to the pathway that produces toxic metabolites. Hepatic necrosis has been known to be a representative side effect of chlorzoxazone (Powere *et al.*, 1986). This toxicity seems to be an idiosyncratic type of reaction because the drug did not have predictable dose-dependent toxicity and this toxicity might be due to the putative toxic metabolites (Zimmerman, 1978). If the P-450 2E1 activity is blocked in the body, the metabolic pathway of chlorzoxazone to 6-hydroxychlorzoxazone may shift to the unknown toxic path-

way and result in the hepatotoxicity.

In conclusion, chlorzoxazone is metabolized to 6-hydroxychlorzoxazone and urinary excretion of this metabolite is dependent on the catalytic activity of P-450 2E1. Determination of the urinary 6-hydroxychlorzoxazone seems to be a useful non-invasive tool to measure *in vivo* P-450 2E1 activity because this drug is metabolized selectively by P-450 2E1.

REFERENCES

- Axoury, F. J. (1982) Double blind comparison of Parafon forte tablet in the treatment of musculoskeletal back conditions. *Curr. Ther. Res.* **26**, 189-197.
- Conney, A. H. and Burns, J. J. (1960) Physiological disposition and metabolic fate of chlorzoxazone in man. *J. Pharmacol. Exp. Ther.* **128**, 340-343.
- Desiraju, R. K., Renzi, N. L., Nakak, R. A., and Ng, K. T. (1983) Pharmacokinetics of chlorzoxazone in humans. *J. Pharmaceutical Sci.* **72**, 991-994.
- Gonzalez, F. J. (1989) The molecular biology of cytochrome P-450s. *Pharmacol. Rev.* **40**, 243-297.
- Guengerich, F. P. (1987) Cytochrome P-450 enzymes and drug metabolism. In *Progress in Drug Metabolism*. Vol 10, pp 1-54. eds by Bridges, J. W., Chasseaud, L. F., and Gibson, G. G.. Taylor & Francis, London.
- Guengerich, F. P. (1989) Analysis and characterization of enzymes. in *Principles and Methods of Toxicology*. ed by Hayes, A. W. Raven Press, New York.
- Guengerich, F. P., Kim, D. H., and Iwasaki, M. (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* **4**, 168-179.
- Hunter, J. L. and Neal, R. A. (1975) Inhibition of hepatic mixed-function oxidase activity *in vitro* and *in vivo* by various thiono-sulfur-containing compounds. *Biochem. Pharmacol.* **24**, 2199-2205.
- Kim, D. H. and Guengerich, F. P. (1990) Formation of the DNA adduct S-[2-(N⁷-guanyl)ethyl]glutathione from ethylene dibromide: effects of modulation of glutathione and glutathione S-transferase levels and lack of a role for sulfation. *Carcinogenesis* **11**, 419-424.
- Koop, D. R., Laethem, C. L., and Schnier, G. C. (1989) Identification of ethanol inducible P-450 isozymes 3a (P-450 IIE1) as a benzene and phenol hydroxylase. *Toxicol. Appl. Pharmacol.* **98**, 278-288.
- Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P., and Yang, C. S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450 IIE1. *Chem. Res. Toxicol.* **3**, 566-573.
- Power, B. J., Cattau, E. L., and Zimmerman, H. J. (1986) Chlorzoxazone hepatotoxic reactions: an analysis of 21 identified or presumed cases. *Arch. Intern. Med.* **146**, 1183-1186.
- Tu, Y. Y. and Yang, C. S. (1983) High-affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting. *Cancer Res.* **43**, 623-629.
- Twele, V. R. and Spitteller, G. (1982) Identifizierung von chlorzoxazon metaboliten in menschlichen urin. *Arzneim. Forsch.* **32**, 759-763.
- Yang, C. S., Yoo, J. S. H., Ishizaki, H., and Hong, J. (1990) Cytochrome P-450 IIE1:

role in nitrosamine metabolism and mechanism of regulation. *Drug Metab. Rev.* **22**, 147-159.

Yoo, J. S. H., Cheung, R. J., Patten, C. J., Wade, D., and Yang, C. S. (1987) Nature of N-nitrosodimethylamine demethylase and its inhibitors. *Cancer Res.* **47**, 3378-3383.

Zimmerman, H. J. (1978) *Hepatotoxicity*. East Norwalk, Conn. Appleton century.