Biochemical Pharmacology, Vol. 33, No. 4, pp. 609–613, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

STEREOSELECTIVE MONOOXYGENATION OF CARCINOSTATIC 1-(2-CHLOROETHYL)-3-(CYCLOHEXYL)-1-NITROSOUREA AND 1-(2-CHLOROETHYL)-3-(*TRANS*-4-METHYLCYCLOHEXYL)-1-NITROSOUREA BY PURIFIED CYTOCHROME P-450 ISOZYMES

DAVID W. POTTER*, WAYNE LEVIN†, DENE E. RYAN†, PAUL E. THOMAS† and DONALD J. REED*‡

* Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, and † Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110, U.S.A.

(*Received* 11 *April* 1983; *accepted* 25 *August* 1983)

Abstract—Three highly purified forms of liver microsomal cytochrome P-450 (P-450a, P-450b and P-450c) from Aroclor 1254-treated rats catalyzed 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (MeCCNU) monooxygenation in the presence of purified NADPH–cytochrome P-450 reductase, NADPH, and lipid. Differences in the regioselectivity of CCNU and MeCCNU monohydroxylation reactions by the cytochrome P-450 isozymes were observed. Cytochrome P-450-dependent monooxygenation of CCNU gave only alicyclic hydroxylation products, but monooxygenation of MeCCNU gave alicyclic hydroxylation products, an α-hydroxylation product on the 2-chloroethyl moiety, and a *trans*-4-hydroxymethyl product. A high degree of stereoselectivity for hydroxylation of CCNU and MeCCNU at the *cis*-4 position of the cyclohexyl ring was demonstrated. All three cytochrome P-450 isozymes were stereoselective in primarily forming the metabolite *cis*-4-hydroxy-*trans*-4-Methyl-CCNU from MeCCNU. The principal metabolite of CCNU which resulted from cytochromes P-450a and P-450b catalysis was *cis*-4-hydroxy CCNU, whereas the principal metabolites from cytochrome P-450c catalysis were the *trans*-3-hydroxy and the *cis*-4-hydroxy isomers. Total amounts of CCNU and MeCCNU hydroxylation with cytochrome P-450b were twice that with hepatic microsomes from Aroclor 1254-treated rats. Catalysis with cytochromes P-450a and P-450c was substantially less effective than that observed with either cytochrome P-450b or hepatic microsomes from Aroclor 1254-treated rats.

Among the growing class of 2-chloroethyl nitrosoureas, CCNU§ and MeCCNU are presently used clinically. Studies on the antineoplastic activity of the nitrosoureas have demonstrated the importance of chemical activation by non-enzymatic breakdown to form the reactive isocyanates and 2-chloroethyl carbonium ions responsible for tumor cell death [1–12]. Additionally, CCNU and MeCCNU have been shown to undergo rapid cytochrome P-450-dependent monooxygenation with hepatic microsomes [13–16]. For a review, see Ref. 17. Hepatic microsomes from phenobarbital (PB)-treated rats principally gave the *cis*-4-hydroxy metabolite of CCNU and MeCCNU [14, 15], whereas hepatic microsomes from 3-methylcholanthrene (3-MC)-treated rats gave *trans*-3-hydroxy and *cis*-4-hydroxy CCNU [14] as the main metabolites. Monooxygenation may occur by homolytic cleavage of the hydrogen with a free radical intermediate formed prior to hydroxylation [18]

Aroclor 1254 is a potent inducer of microsomal cytochrome P-450 with mixed substrate specificity similar to that observed by combined PB and 3-MC treatment of rats [19]. Purification and immunoquantitation studies [20–22] have demonstrated that the major PB-induced cytochrome P-450 (P-450b) and the major 3-MC-induced cytochrome P-450 (P-450c)

‡ Author to whom all correspondence should be addressed.

§ Abbreviations: CCNU, 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; *cis*-3-hydroxy-Methyl-CCNU, 1-(2-chloroethyl)-3-(*cis*-3-hydroxy-*trans*-4-methylcyclohexyl)-1-nitrosourea; *trans*-3-hydroxy-Methyl-CCNU, 1-(2-chloroethyl)-3-(*trans*-3-hydroxy-*trans*-4-methylcyclohexyl)-1-nitrosourea; *cis*-4-hydroxy-Methyl-CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxy-*trans*-4-methylcyclohexyl)-1-nitrosourea; *trans*-4-hydroxy-*cis*-4-Methyl-CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxy-*cis*-4-methylcyclohexyl)-1-nitrosourea; *trans*-4-hydroxy-Methyl-CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxymethylcyclohexyl)-1-nitrosourea; α-hydroxy-Methyl-CCNU, 1-(1-hydroxy-2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; *cis*-2-hydroxy CCNU, 1-(2-chloroethyl)-3-(*cis*-2-hydroxycyclohexyl)-1-nitrosourea; *trans*-2-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-2-hydroxycyclohexyl)-1-nitrosourea; *trans*-3-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-3-hydroxycyclohexyl)-1-nitrosourea; *cis*-3-hydroxy CCNU, 1-(2-chloroethyl)-3-(*cis*-3-hydroxycyclohexyl)-1-nitrosourea; *cis*-4-hydroxy CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea; *trans*-4-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; AC, Aroclor 1254; 3-MC, 3-methylcholanthrene; and PB, phenobarbital.

Table 1. Comparison of CCNU metabolite formation with microsomal and purified enzyme preparations

	Percent of total CCNU monohydroxylated derivatives						
	Microsomes				P-450 isozymes		
Metabolite	Control*	PB†	3-MC*	AC	P-450a	P-450b	P-450c
cis-2-hydroxy	ND‡	ND	ND	ND	1	0	3
trans-2-hydroxy	ND	2	ND	ND	1	1	4
trans-3-hydroxy	39	6	40	5	17	2	49
cis-3-hydroxy	30	20	25	18	4	20	1
cis-4-hydroxy	21	63	30	72	77	73	43
trans-4-hydroxy	9	6	5	6	ND	5	ND

* See Ref. 14.
† See Ref. 29.
‡ Not detected.

are both induced by treatment of rats with Aroclor 1254. A minor cytochrome P-450 isozyme, cytochrome P-450a, which is modestly induced by Aroclor 1254, was also purified.

This report describes the monooxygenation of CCNU and MeCCNU catalyzed by cytochromes P-450a, P-450b, and P-450c purified from the hepatic microsomes of Aroclor 1254-treated rats. Cytochrome P-450b was catalytically more active than cytochromes P-450a and P-450c for the metabolism of CCNU and MeCCNU. Microsomal and purified cytochrome P-450s primarily catalyzed the cyclohexyl ring monohydroxylation of CCNU and MeCCNU to give *cis*-4-hydroxy metabolites, with the exception of CCNU monooxygenation catalyzed by cytochrome P-450c which gave *trans*-3-hydroxy and *cis*-4-hydroxy CCNU as the major metabolites.

METHODS

Purification of cytochrome P-450. Hepatic microsomes from Aroclor 1254-pretreated Long Evans rats (55–60 g, Blue Spruce Farms, Altamont, NY) were prepared as previously described [23]. Cytochromes P-450a, P-450b, and P-450c were purified from microsomal preparations by described methods [20] to a specific content of 12–16 nmoles/mg protein. Protein was determined by the method of Lowry *et al.* [24], and the concentration of cytochrome P-450 was determined according to Omura and Sato [25] from the CO-reduced difference spectrum with an extinction coefficient of 91 mM^{-1} cm^{-1}.

NADPH–cytochrome P-450 reductase. By combining the methods of Dignam and Strobel [26], and Yasukochi and Masters [27], the NADPH–cytochrome P-450 reductase was purified to a specific activity of 35.0 to 40.0 units/mg protein. NADPH–cytochrome P-450 reductase activity was assayed at 22° by following the rate of cytochrome *c* reduction (1 unit equals one μmole of cytochrome *c* reduced/min) by the method of Phillips and Langdon [28].

Incubation procedure. Reaction mixtures of 1.0 ml of the reconstituted system contained NADPH–cytochrome P-450 reductase (1.0 unit), cytochromes P-450a, P-450b and P-450c (0 to 0.15 nmole), dilauroylphosphatidylcholine (0–30 μg), potassium phosphate buffer, pH 7.4 (100 μmoles), and CCNU or MeCCNU (500 nmoles) in 10 μl of dimethyl sulfoxide (DMSO). Reactions were initiated by the addition of NADPH (1.0 μmole), and the mixture was placed in a reciprocal shaker water bath at 37°. The addition of cold ether after 10 min terminated the reactions. Metabolites were extracted and analyzed by liquid chromatography as previously described [13–15].

RESULTS

CCNU was metabolized by hepatic microsomes from Aroclor 1254-treated rats to yield *trans*-3-hydroxy CCNU, *cis*-3-hydroxy CCNU, *cis*-4-hydroxy CCNU, and *trans*-4-hydroxy CCNU in the presence of NADPH. Metabolite formation was specific for the cyclohexyl ring region. The *cis*-4 position was hydroxylated more rapidly than the *trans*-4-position. The *cis*-4-hydroxy CCNU was the major hydroxylation product; the *trans*-4-hydroxy CCNU isomer was a minor hydroxylation product. The principal carbon-3-monooxygenation product was *cis*-3-hydroxy CCNU with an observed rate of formation nearly 4-fold greater than the *trans*-3-isomer. Thus, the preferred sites of attack by hepatic microsomes from Aroclor 1254-treated rats were the *cis* over the *trans* and carbon-4 over carbon-3 (Table 1).

In descending order of metabolic rate, these microsomes metabolized MeCCNU to yield primarily *cis*-4-hydroxy-*trans*-4-Methyl-CCNU, and lesser amounts of *cis*-3-hydroxy-*trans*-4-Methyl-CCNU, α-hydroxy-*trans*-4-Methyl-CCNU, *trans*-4-hydroxy-*cis*-4-Methyl-CCNU, *trans*-4-hydroxymethyl-CCNU and *trans*-3-hydroxy-*trans*-4-methyl-CCNU. The overall rate of MeCCNU monooxygenation was 84% compared to that of CCNU with a similar specificity for cyclohexyl ring monohydroxylation (Table 2). However, MeCCNU was also hydroxylated on the methyl and the 2-chloroethyl moieties which indicates that the microsomes from Aroclor 1254-treated rats were less regioselective for MeCCNU than for CCNU.

Figure 1 shows the effect of lipid concentration on the rates of CCNU and MeCCNU cyclohexyl ring *cis*-4 hydroxylation catalyzed by purified cytochromes P-450a, P-450b and P-450c and NADPH–cytochrome P-450 reductase. Catalytic activity was usually greater when purified enzymes were reconstituted with dilauroylphosphatidylcholine, particularly

Table 2. Comparison of MeCCNU metabolite formation with microsomal and purified enzyme preparations

	Percent of total MeCCNU monohydroxylated derivatives					
	Microsomes			P-450 isozymes		
Metabolite	Control*	PB*	AC	P-450a	P-450b	P-450c
trans-3-hydroxy	11	4	3	18	4	ND†
α-hydroxy	7	18	12	9	9	2
cis-4-hydroxy	16	41	59	66	63	90
cis-3-hydroxy	13	14	15	3	14	2
trans-4-hydroxy	nd‡	11	6	4	9	3
trans-4-hydroxy-methyl	52	12	5	ND	2	3

* See Ref. 15. † Not detected. ‡ Not determined.

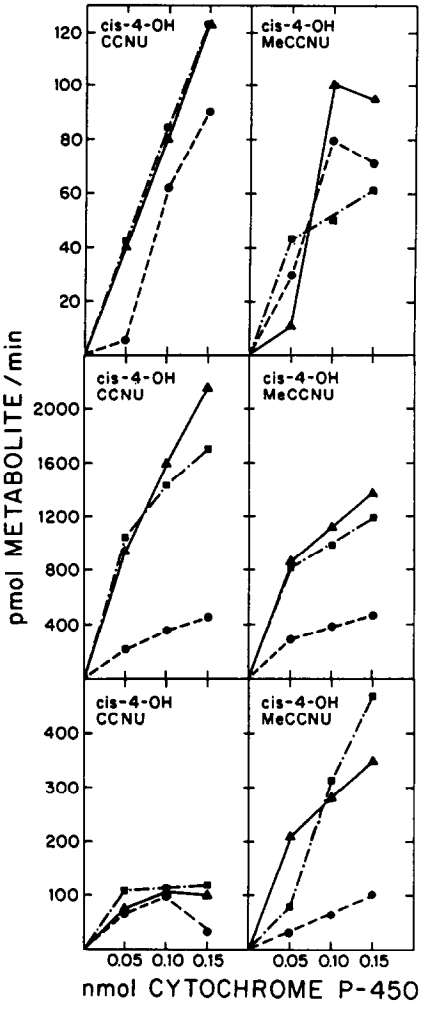

Fig. 1. Effect of dilauroylphosphatidylcholine and purified cytochrome P-450 isozymes on *cis*-4-hydroxy CCNU (left three panels) and *cis*-4-hydroxy-*trans*-4-methyl-CCNU (right three panels) formation from CCNU and MeCCNU respectively. Reaction mixtures of 1.0 ml contained 0.05 to 0.15 nmole of cytochromes P-450a (upper two panels), P-450b (middle two panels) or P-450c (lower two panels) and 1.0 unit of NADPH–cytochrome P-450 reductase, reconstituted without (●---●), or with 10 μg (▲—▲) or 30 μg (■···■) of dilauroylphosphatidylcholine. Other conditions are described in Methods.

at higher cytochrome P-450 concentrations. As indicated in the figure, the effect of lipid concentration was less profound with cytochromes P-450a and P-450c than with cytochrome P-450b when the catalytic rate of *cis*-4-hydroxy CCNU formation was increased 5-fold in the presence of lipid (10 μg).

In the reconstituted system containing purified cytochrome P-450 and NADPH–cytochrome P-450 reductase, CCNU metabolism varied markedly with the use of different isozymes (Fig. 2). CCNU cytochrome P-450b-dependent hydroxylation was approximately twice that of the Aroclor 1254-induced microsomes, twenty times that of cytochrome P-450a, and eight times that of cytochrome P-450c monooxygenation activity. The ratios of metabolite formation which resulted from CCNU metabolism by cytochrome P-450b and Aroclor 1254-induced microsomes were almost indistinguishable except that, in the former case, trace amounts of *cis*-2-hydroxy CCNU and *trans*-2-hydroxy CCNU were formed.

Cytochrome P-450a was the least effective cytochrome P-450 isozyme with respect to CCNU

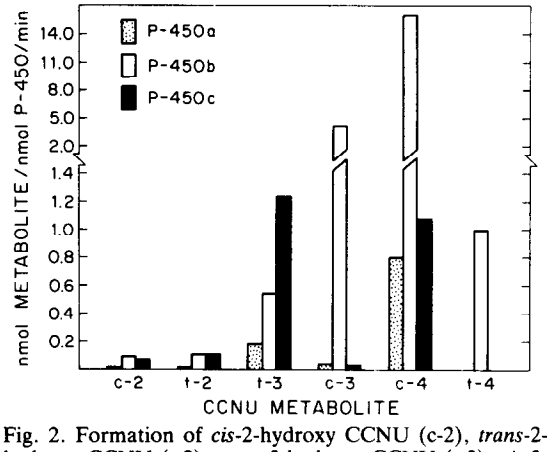

Fig. 2. Formation of *cis*-2-hydroxy CCNU (c-2), *trans*-2-hydroxy CCNU (t-2), *trans*-3-hydroxy CCNU (t-3), *cis*-3-hydroxy CCNU (c-3), *cis*-4-hydroxy CCNU (c-4), and *trans*-4-hydroxy CCNU (t-4), from CCNU, catalyzed by purified cytochromes P-450a, P-450b, and P-450c. Reaction mixtures of 1.0 ml contained cytochrome P-450 (0.1 nmole) and NADPH–cytochrome P-450 reductase (1.0 unit) reconstituted with dilauroylphosphatidylcholine (10 μg). Other conditions are described in Methods.

metabolism. Although metabolism of monooxygenation was less effective with cytochrome P-450a than with cytochrome P-450c, both cytochrome isozymes were highly selective in attack at the *trans*-3 and *cis*-4 positions. The major monohydroxylated CCNU metabolites with cytochrome P-450c were *trans*-3-hydroxy and *cis*-4-hydroxy CCNU. The *trans*-3- and *cis*-4-hydroxy CCNU metabolites were formed at nearly equal amounts and together accounted for most of the cytochrome P-450c metabolic activity (Table 1). Cytochrome P-450a-catalyzed formation of *cis*-4-hydroxy and *trans*-3-hydroxy CCNU accounted for 77 and 17% of the total metabolites, respectively.

The principal MeCCNU metabolite formed in the reconstituted system by purified cytochromes P-450a, P-450b and P-450c was *cis*-4-hydroxy-*trans*-4-Methyl-CCNU. Catalysis of monooxygenation of MeCCNU, like CCNU, in the presence of purified cytochrome P-450b was twice as rapid as with microsomes from Aroclor 1254-treated rats (Fig. 3). However, the metabolic profile with cytochrome P-450b was also similar to that observed with microsomes from Aroclor 1254-treated rats. As with CCNU, the *trans*-3-hydroxy-Methyl-CCNU isomer was formed by cytochrome P-450a as a minor second metabolite. In contrast, no more than 10% of the cytochrome P-450c-dependent MeCCNU hydroxylation activity was observed at sites other than the cyclohexyl ring *cis*-4 position. Cytochromes P-450a and P-450c catalyzed MeCCNU hydroxylation at rates slightly greater than CCNU.

DISCUSSION

The principal hydroxy metabolites of CCNU were *trans*-3- and *cis*-4-hydroxy CCNU with cytochrome P-450c, and *cis*-4-hydroxy CCNU with cytochromes P-450a and P-450b. All three purified cytochrome P-450 isozymes metabolized MeCCNU predominantly at the *cis*-4-cyclohexyl ring position. When cytochrome P-450 isozymes and NADPH–cytochrome P-450 reductase were reconstituted with dilauroylphosphatidylcholine, catalysis was more efficient than when lipid was not included.

Traditionally, the cytochrome P-450 monooxygenation of foreign substances is regarded to be a detoxification reaction. However, it is well established that cytochrome P-450 activates certain compounds to give metabolites that are more toxic or carcinogenic than the parent compound. The polycyclic aromatic hydrocarbons, as an example, undergo cytochrome P-450 activation to become ultimate carcinogens and mutagens [30].

Monooxygenation of the cyclohexyl nitrosoureas give metabolites that are not truly activated or inactivated [31]. The hydroxy metabolites of CCNU and MeCCNU exhibit antitumor activity as do the parent nitrosoureas. Since cytochrome P-450 monooxygenation is rapid, the hydroxylated metabolites of CCNU and MeCCNU are considered to be the principal *in vivo* metabolites that are responsible for the observed therapeutic activity [32]. Although the advantages of monooxygenation are difficult to assess, a comparison of physical and chemical properties of CCNU metabolites indicates that some metabolites may have a therapeutic advantage over CCNU itself [31]. In particular, *trans*-2-hydroxy CCNU showed promise. But as studies with microsomes [14, 16, 29] and purified enzyme preparations (this manuscript) demonstrate, *trans*-2-hydroxy CCNU formation is barely detectable. Thus, the advantages or disadvantages of monooxygenation remain obscure.

With the recent attention given to nitrosourea denitrosation [33, 34], one advantage of hydroxylation may be that metabolites are less susceptible to denitrosation. The denitrosation of *cis*-4-hydroxy CCNU was 65% and *trans*-4-hydroxy CCNU was 50% of the rate observed with CCNU and hepatic microsomes from PB-treated rats.* A slower rate of denitrosation could change the therapeutic activity, since loss of the nitroso group renders the nitrosourea inert.

Although microsomal enzyme induction and inhibition indicated potential cytochrome P-450 isozyme specificity, the necessity to demonstrate the oxidation of nitrosoureas with purified cytochrome P-450 remained to be determined. CCNU metabolite formation, first elucidated by May *et al.* [13, 14] using microsomes from PB- or 3-MC-treated rats, was useful in anticipating the ratio of metabolite formation with cytochromes P-450b and P-450c, yet unequivocal assignment of isozyme specificity was not possible (Table 1). Direct analogy between microsomes and the purified system cannot be made since a large number of isozymes are known to exist and isozyme composition may vary depending on the age and the genetic strain of animals. Although microsomes from PB-treated rats and purified cytochrome P-450b primarily formed *cis*-4-hydroxy CCNU, the microsomes gave a larger proportion of *trans*-3-hydroxy CCNU than cytochrome P-450b. With microsomes from 3-MC-treated rats and purified P-450c, *trans*-3- and *cis*-4-hydroxy CCNU were the predominant metabolites. In contrast, *cis*-3-hydroxy CCNU accounted for 25% of the metabolites formed with microsomes from 3-MC-treated rats;

* D. W. Potter and D. J. Reed, unpublished data.

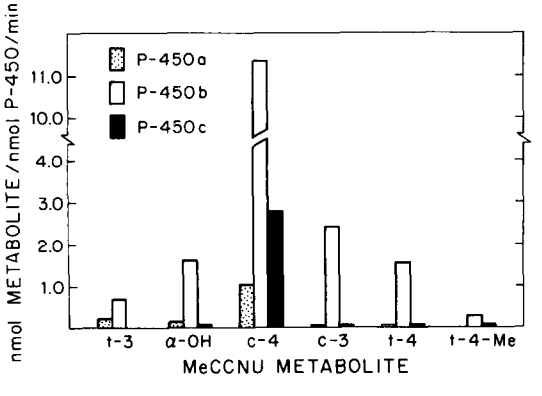

Fig. 3. Formation of *trans*-3-hydroxy-*trans*-4-Methyl-CCNU (t-3), α-hydroxy-*trans*-4-Methyl-CCNU (α-OH), *cis*-4-hydroxy-*trans*-4-Methyl-CCNU (c-4), *cis*-3-hydroxy-*trans*-4-Methyl-CCNU (c-3), *trans*-4-hydroxy-*cis*-4-Methyl-CCNU (t-4), and *trans*-4-hydroxymethyl-CCNU (t-4-Me), from MeCCNU, catalyzed by cytochromes P-450a, P-450b, and P-450c as described in Methods.

only trace amounts were formed with cytochrome P-450c.

Differences in the stereoselectivity of MeCCNU metabolism by microsomal and purified enzymes were also demonstrated. Monohydroxylation studies performed by others [15] have been included in Table 2 for comparison. The *trans*-4-hydroxymethyl-CCNU metabolite is formed primarily with liver microsomes from untreated rats, while *cis*-4-hydroxy-Methyl-CCNU is the major metabolite with microsomes from PB-treated rats [15]. Of the three isozymes analyzed here (cytochromes P-450a, P-450b, and P-450c), none exhibited specificity towards the hydroxylation of the cyclohexyl methyl group. Therefore, the data suggest the involvement of at least one other cytochrome P-450 isozyme with stereoselective activity for the methyl moiety of the MeCCNU cyclohexyl ring.

Acknowledgement—This research was aided in part by Grant CH-109 from the American Cancer Society.

REFERENCES

1. J. A. Montgomery, R. James, G. S. McCaleb and T. P. Johnson, *J. med. Chem.* **10**, 668 (1967).
2. D. J. Reed, H. E. May, R. B. Boose, K. M. Gregory and M. A. Beilstein, *Cancer Res.* **35**, 568 (1975).
3. M. Colvin, J. W. Cowens, R. B. Brundett, B. S. Kramer and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **60**, 515 (1974).
4. C. J. Chen, S. Fugimura, D. Grunberger and I. B. Weinstein, *Cancer Res.* **32**, 22 (1972).
5. H. W. Kann, Jr., K. W. Kohn, L. Widerlife and D. Guillion, *Cancer Res.* **34**, 1982 (1974).
6. P. V. Woolley, III, R. L. Dion, K. W. Kohn and V. H. Bono, Jr., *Cancer Res.* **36**, 1470 (1976).
7. J. R. Babson, D. J. Reed and M. A. Sinkey, *Biochemistry* **16**, 1584 (1977).
8. J. R. Babson and D. J. Reed, *Biochem. biophys. Res. Commun.* **83**, 754 (1978).
9. B. S. Kramer, C. C. Fenselau and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **56**, 783 (1974).
10. B. J. Bowdon and G. P. Wheeler, *Proc. Am. Ass. Cancer Res.* **12**, 67 (1971).
11. K. W. Kohn, *Cancer Res.* **37**, 1450 (1977).
12. J. W. Lown, L. W. McLaughlin and Y-M. Chang, *Bioorg. Chem.* **7**, 97 (1978).
13. H. E. May, R. Boose and D. J. Reed, *Biochem. biophys. Res. Commun.* **57**, 426 (1974).
14. H. E. May, R. Boose and D. J. Reed, *Biochemistry* **14**, 4723 (1975).
15. H. E. May, S. J. Kohlhepp, R. B. Boose and D. J. Reed, *Cancer Res.* **39**, 762 (1979).
16. J. Hilton and M. D. Walker, *Biochem. Pharmac.* **24**, 2153 (1975).
17. D. J. Reed, in *Nitrosoureas, Current Status and New Developments* (Eds. A. W. Prestayko, S. T. Crooke, L. H. Baker, S. K. Carter and P. S. Schein), p. 51. Academic Press, London (1981).
18. D. W. Potter and D. J. Reed, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Eds. M. J. Coon, R. W. Conney, H. V. Gelboin, J. R. Gillette and P. J. O'Brien), p. 371. Academic Press, New York (1980).
19. A. P. Alvares, D. R. Bickers and A. Kappas, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1321 (1973).
20. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
21. W. Levin, L. H. Botelho, P. E. Thomas and D. E. Ryan, in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* (Eds. M. J. Coon, R. W. Conney, H. V. Gelboin, J. R. Gillette and P. J. O'Brien), p. 47. Academic Press, New York (1980).
22. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **256**, 1047 (1981).
23. D. E. Ryan, P. E. Thomas and W. Levin, *Molec. Pharmac.* **13**, 521 (1977).
24. O. H. Lowry, H. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
26. J. D. Dignam and H. W. Strobel, *Biochem. biophys. Res. Commun.* **63**, 845 (1975).
27. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
28. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
29. P. B. Farmer, A. B. Foster, M. Jarman, M. R. Oddy and D. J. Reed, *J. med. Chem.* **21**, 514 (1978).
30. W. Levin, A. W. Wood, R. Chang, D. Ryan, P. Thomas, H. Yagi, D. Thakker, K. Vyas, C. Boyd, S-Y. Chu, A. H. Conney and D. M. Jerina, *Drug Metab. Rev.* **13**, 543 (1982).
31. G. P. Wheeler, T. P. Johnston, B. J. Bowden, G. S. McCaleb, D. L. Hill and J. A. Montgomery, *Biochem. Pharmac.* **26**, 2331 (1977).
32. D. J. Reed, in *Synthesis and Properties of Antitumor and Interferon-Inducing Drugs* (Eds. G. Butler and R. Ottenbrite), pp. 177–202. Marcel Dekker, New York (1982).
33. D. W. Potter and D. J. Reed, *Archs Biochem. Biophys.* **216**, 158 (1982).
34. D. W. Potter and D. J. Reed, *J. biol. Chem.* **258**, 6906 (1983).