Biochemical Pharmacology, Vol. 29, pp. 2031–2037

0006-2952/80/0715-2031 $02.00/0

EFFECTS OF PHENOBARBITAL AND β-NAPHTHOFLAVONE ON THE ACTIVATION OF CYCLOPHOSPHAMIDE TO MUTAGENIC METABOLITES *IN VITRO* BY LIVER AND KIDNEY FROM MALE AND FEMALE RATS

BARBARA F. HALES and RANJANA JAIN

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, H3G 1Y6, Canada

(*Received* 5 *November* 1979; *accepted* 29 *January* 1980)

Abstract—The activation of cyclophosphamide to metabolites that are mutagenic to *Salmonella typhimurium* TA 1535 by liver microsomes and kidney S9 fractions from male and female rats was studied. In addition, the effect of pretreatment with inducers of cytochromes P-450, phenobarbital and β-naphthoflavone on the activation of cyclophosphamide by these tissues was evaluated. The activation of cyclophosphamide to mutagenic metabolites with microsomes from male rat liver was three times that obtained with microsomes from female rat liver. Whereas pretreatment of either male or female rats with phenobarbital increased hepatic activation of cyclophosphamide to mutagenic metabolites about 10-fold, the increase in cytochrome P-450 content was only about 2-fold. β-Naphthoflavone pretreatment of either male or female rats decreased hepatic activation of cyclophosphamide by one-half despite an increase of 1.4-fold in hepatic cytochrome P-450 content. Kidney S9 fractions from male rats had one-third to one-half the ability of liver microsomes to activate cyclophosphamide to mutagenic metabolites; however, no enzymatic activation of cyclophosphamide to mutagenic metabolites by female rat kidney was noted. Neither phenobarbital nor β-naphthoflavone pretreatment altered the metabolic activation of cyclophosphamide to mutagenic metabolites by kidney fractions from either sex. These results demonstrate that enzymatic activation of cyclophosphamide to mutagenic metabolites differs markedly between the sexes, between different tissues (liver vs kidney), and in response to inducing agents.

Cyclophosphamide is used widely both as an antineoplastic agent and as an immunosuppressive agent. Active metabolites of cyclophosphamide, rather than the parent compound, have alkylating and mutagenic activities and cause cytotoxic reactions, mutations, chromosomal aberrations and oncogenic transformation [1–6]. The activation of cyclophosphamide to therapeutic and toxic metabolites is catalyzed by a cytochrome P-450 mono-oxygenase system [2]. It is now well established that there are multiple cytochromes P-450, each differing with respect to substrate specificity, carbon monoxide difference spectrum, response to a variety of inducing agents, and sensitivity to different inhibitors [7–9]. We established previously that the production of mutagenic metabolites of cyclophosphamide by male rat liver is enhanced by phenobarbital pretreatment and is decreased by pretreatment with β-naphthoflavone [10]. Thus, the form of cytochrome P-450 involved in the production of mutagenic metabolites of cyclophosphamide by microsomes from male rat liver is responsive to induction by phenobarbital rather than by β-naphthoflavone [10].

Sex differences have been observed with respect to certain hepatic cytochrome P-450 catalyzed reactions [11–14]. Microsomes from male rat liver produced alkylating metabolites of cyclophosphamide at a rate six to seven times faster than that produced by microsomes of female rat liver [2]. However, no such proportional differences in the *in vivo* metabolism of cyclophosphamide to alkylating metabolites by male and female rats have been found [2]. In contrast, the activation of cyclophosphamide to mutagenic metabolites has not been studied extensively [4, 6], and no comparison of such activities between male and female has been reported.

One interpretation of the lack of correlation between *in vivo* metabolism of cyclophosphamide to alkylating metabolites in male and female rats and hepatic *in vitro* metabolism is that cyclophosphamide is also converted to alkylating metabolites in extrahepatic tissues. Both cytochrome P-450 and mono-oxygenase activity are present in a variety of tissues other than liver [15, 16]. Several laboratories compared different tissues with respect to their abilities to activate cyclophosphamide to alkylating metabolites *in vitro*. Although most alkylating activity was found in the liver, Brock and Hohorst [17] and Kondo and Muragishi [18] reported the existence in rat kidney of enzymes catalyzing the activation of cyclophosphamide. Other investigators [2, 19], however, have not been able to detect such activation in kidney. Metabolites of cyclophosphamide (specifically acrolein) are excreted in the urine and cause cystitis [20]; thus, it was not surprising that nephrectomy prevented this cystitis [21]. Yet, Levine and Sowinski [21] found that anuria produced a greater increase in cyclophosphamide lesions in brain, eye

and spleen than did nephrectomy. Consequently, it was hypothesized that the kidneys play a role in the *in vivo* production of cytotoxic metabolites of cyclophosphamide.

The data presented below demonstrate that there is a sex difference with respect to liver activation of cyclophosphamide to mutagenic metabolites. Both male and female hepatic activation can be induced by phenobarbital, but not by β-naphthoflavone. The activation of cyclophosphamide to mutagenic metabolites can also be catalyzed by male kidney S9 fractions. Renal activation of cyclophosphamide is not induced by pretreatment with either phenobarbital of β-naphthoflavone.

MATERIALS AND METHODS

Chemicals. Cyclophosphamide was purchased from Koch–Light Laboratories Ltd., Colnbrook, England; β-naphthoflavone from ICN Pharmaceuticals, Inc., Plainview, NY, U.S.A.; and phenobarbital from Allen & Hanburys, Toronto, ON, Canada. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and bovine serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals. Male and female Sprague–Dawley rats (175–200 g), obtained from Canadian Breeding Laboratories (St. Constant, Quebec), were housed on pine chips in the McIntyre Animal Center (McGill University, Montreal, Quebec), and given Purina rat chow and water *ad lib.* for at least 1 week before the initiation of treatments. Each group of animals consisted of five rats. Induced rats were treated with phenobarbital or β-naphthoflavone according to established procedures for induction of the hepatic mono-oxygenase system [22]. The phenobarbital-pretreated rats received 0.1% of the drug in the drinking water for 1 week before being killed; control rats were not treated. The β-naphthoflavone-pretreated animals received β-naphthoflavone intraperitoneally (40 mg/kg) in corn oil (10 mg/ml) on day 1, day 2 and day 3 before being killed on day 5; control rats received corn oil on these days.

Tissue preparation. Rats were decapitated. The excised tissues from each group (five rats) were pooled and homogenized using a Potter–Elvehjem homogenizer in ice-cold 0.15 M KCl (3 ml/g wet weight of tissue). Homogenates were centrifuged for 10 min at 9000 *g* and the pellet was discarded. The supernatant fraction (S9) was distributed in 3-ml portions in small polypropylene tubes, frozen in liquid nitrogen, and stored at −80°. Since liver from phenobarbital-pretreated male rats is the tissue that can best activate cyclophosphamide to mutagenic metabolites, it was used to determine the effects of

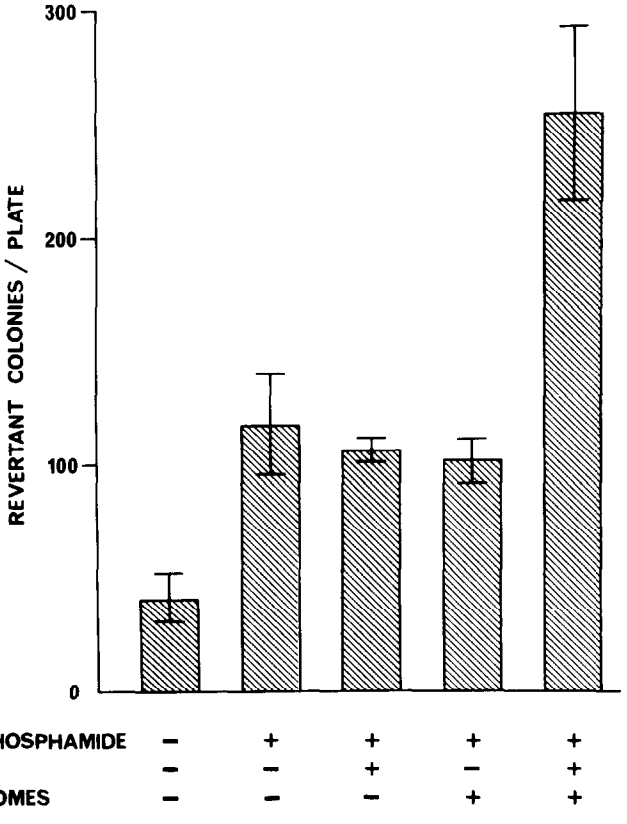

Fig. 1. Requirements for the enzymatic activation of cyclophosphamide to mutagenic metabolites. *S. typhimurium* TA 1535 (0.1 ml), cyclophosphamide (500 μg in 0.1 ml) and 'mix' (0.5 ml) containing the designated combination of NADPH-generating system and microsomal fraction from control male rat liver (0.1 ml/plate) were mixed rapidly in molten top agar at 45° and poured on minimal glucose agar plates. Values are means ± S.E.M. (N = 5).

freezing and storage on the activation of cyclophosphamide. No loss of ability to activate cyclophosphamide to mutagenic metabolites was observed after storage for up to 6 weeks; because storage for longer time periods did result in a decrease in activity, enzyme preparations were assayed in less than 6 weeks. To prepare the microsomal fraction, the S9 fraction was defrosted and centrifuged at 105,000 *g* for 60 min, and the pellet was resuspended in the original volume of cold 0.15 M KCl.

Cytochrome P-450 content. Cytochrome P-450 was assayed, by its carbon monoxide difference spectrum after reduction with dithionite according to the procedure of Omura and Sato [23], with the use of a Beckman model 35 spectrophotometer. An extinction coefficient of 91 $mM^{-1} cm^{-1}$ between 450 and 490 nm was assumed. The protein concentration was determined by the method of Lowry *et al.* [24], using bovine serum albumin as a standard.

Mutagenicity assay. Salmonella typhimurium strain TA 1535 (provided by Dr. Bruce Ames) was used to assay for mutagenic activity. TA 1535 is a histidine-requiring auxotroph that is reverted to prototrophy by mutagens that cause DNA base-pair substitutions. The plate incorporation assay was performed according to the procedures of Ames *et al.* [22]. The NADPH-generating system contained 4 mM NADP, 5 mM glucose-6-phosphate and 2 units/ml of glucose-6-phosphate dehydrogenase. All assays were done in duplicate. After incubation for 2 days at 37°, the colonies were counted. The spontaneous reversion rate (no cyclophosphamide) was similar to that reported by Ames *et al.* [22] for TA 1535 (Fig. 1). The inclusion of enzyme fractions from liver or kidney of male or female rats did not alter this spontaneous reversion rate. Cyclophosphamide alone (500 μg/plate) induced mutations in TA 1535 (Fig. 1). This mutagenic response was not changed by the addition of either the NADPH-generating system alone or the microsomal fraction alone; however, the number of revertants per plate was significantly increased ($P < 0.01$) by the addition of both the NADPH-generating system and the microsomal fraction from control male rat liver (Fig. 1). The mutagenic response in TA 1535 induced by cyclophosphamide in the absence of an enzyme fraction has been subtracted in subsequent experiments, thus allowing the measurement of the enzymatic activation of cyclophosphamide. As described previously [10], this mutagenicity assay was linear with both cyclophosphamide concentration and amount of male liver enzyme (S9 fraction or microsomal fraction).

Statistical analysis. Data were analyzed on a Hewlett–Packard 9830 calculator according to procedures described by Snedecor and Cochran [25]. Reversion rates obtained with increasing amounts of enzyme were compared to the rates obtained with cyclophosphamide alone (no enzyme), using multiple range and/or linear regression analyses as applicable.

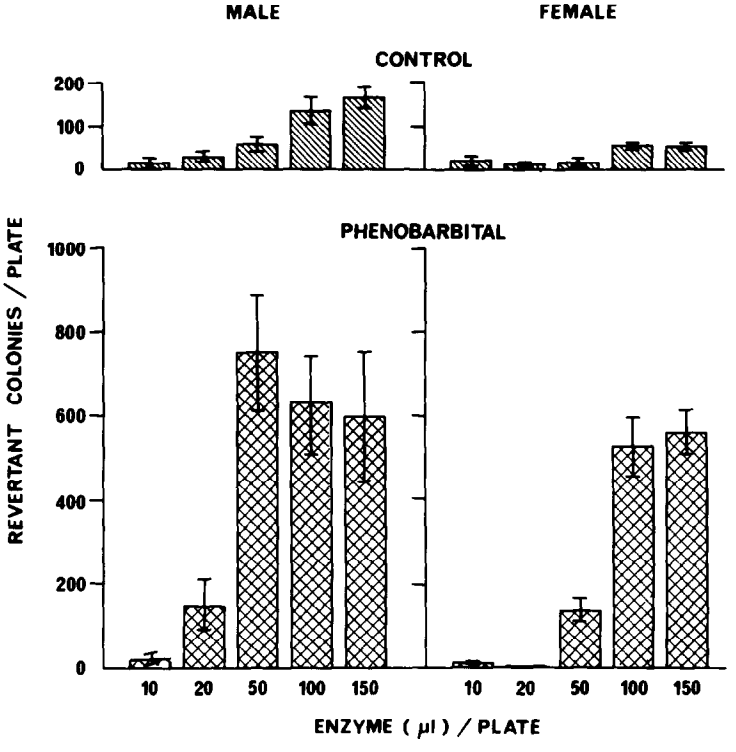

Fig. 2. Comparison of the activation of cyclophosphamide to mutagenic metabolites by untreated and phenobarbital-pretreated male and female rat liver microsomes. *S. typhimurium* TA 1535 (0.1 ml), cyclophosphamide (500 μg in 0.1 ml) and mix (NADPH-generating system and various amounts of resuspended microsomes from control or phenobarbital-induced rat liver, 0.5 ml) were mixed rapidly with molten agar at 45° and plated. Values are means ± S.E.M. (control male, N = 8; phenobarbital male, N = 6; control female, N = 4; phenobarbital female, N = 3).

Table 1. Cytochrome P-450 content in liver and kidney microsomes from male and female rats

Tissue	Cytochrome P-450 content* (nmoles/mg microsomal protein)			
	Treatment group			
	Control	Phenobarbital	Corn oil	β-Naphthoflavone
Liver				
Male	0.55 ± 0.04	1.38 ± 0.03	0.65 ± 0.05	0.99; 0.77
Female	0.56 ± 0.06	1.01 ± 0.02	0.37 ± 0.03	0.53 ± 0.06
Kidney				
Male	0.07; 0.07	0.11 ± 0.03	0.10 ± 0.02	0.07; 0.07
Female	0.07; 0.07	0.06 ± 0.01	0.09; 0.09	0.11; 0.12

* Cytochrome P-450 content was measured as described in the text. Values represent either the mean ± S.E.M. for N = 3 or individual values.

Enzyme fractions from different sources were compared, with respect to activation of cyclophosphamide, by two-way analysis of variance.

RESULTS

Hepatic activation of cyclophosphamide to mutagenic metabolites by control and phenobarbital-pretreated male and female rats is illustrated in Fig. 2. Activation of cyclophosphamide to mutagenic metabolites by liver microsomes from untreated male rats was significantly greater ($P < 0.001$ with 100 μl/plate, or more) than the mutagenic response to cyclophosphamide alone (no enzyme), and the mean was linearly related to the amount of microsomes used ($r = 0.98$). As reported previously [10], this activity was induced 4- to 14-fold by phenobarbital pretreatment, whereas cytochrome P-450 concentration (Table 1) was induced only 2.6-fold. With phenobarbital-induced microsomes from male liver, maximal enzyme activity was obtained with 50 μl of resuspended microsomes/plate ($P < 0.001$). Increasing the amount of enzyme above 50 μl of microsomes/plate did not increase significantly the number of revertant colonies/plate ($P > 0.10$).

Activation of cyclophosphamide to mutagenic metabolites by microsomes from female liver was also significantly greater ($P < 0.01$) than that with cyclophosphamide alone, at amounts of microsomal fraction above 100 μl/plate. However, this activation by microsomes from female rat liver was approximately one-third of that by microsomes from male liver. Despite this sex difference in activation of cyclophosphamide, there was no difference in cytochrome P-450 content between microsomes from

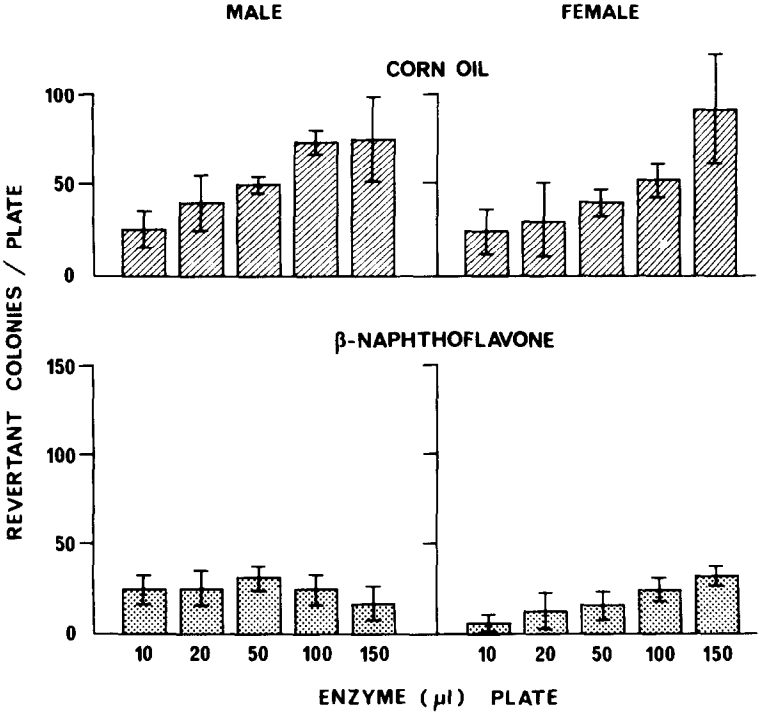

Fig. 3. Comparison of the activation of cyclophosphamide to mutagenic metabolites by corn oil and β-naphthoflavone-pretreated male and female rat liver microsomes. Experiments were done as described in the legend to Fig. 2. Values are means ± S.E.M. (N = 3 for all groups except for the β-naphthoflavone male where N = 6).

male and female liver (Table 1). Phenobarbital pretreatment of female rats induced hepatic activation of cyclophosphamide 10- to 13-fold, increasing activity to the same peak level as was observed for phenobarbital-induced male microsomes. Hepatic cytochrome P-450 content was induced 1.8-fold in the female rats (Table 1). Maximal activation of cyclophosphamide was not observed until 100 μl of resuspended microsomes/plate from female liver was used; this is in contrast to the lower amount (50 μl/plate) of microsomes needed to reach maximal activation in the male.

The effect of pretreatment with corn oil or β-naphthoflavone in corn oil on the capacity of liver microsomes to activate cyclophosphamide to mutagenic metabolites is shown in Fig. 3. The mean activation of cyclophosphamide by microsomes from corn oil-pretreated male rats was linearly related ($r = 0.98$) to the amount of microsomes and significantly greater ($P < 0.05$) than that without enzyme (100 μl/plate or more). Pretreatment of male rats with β-naphthoflavone decreased the hepatic activation of cyclophosphamide to mutagenic metabolites. Using as much as 150 μl/plate of microsomes from β-naphthoflavone-pretreated male rats, there was no significant ($P > 0.05$) enzymatic activation of cyclophosphamide; however, the cytochrome P-450 content of these microsomes was increased 1.4-fold above the corn oil-treated control microsomes (Table 1). Enzymatic activation by hepatic microsomes from corn oil-pretreated female rats was significantly greater ($P < 0.01$) than that with cyclophosphamide alone (150 μl of microsomal fraction/plate). Again, β-naphthoflavone pretreatment of female rats appeared to decrease activation of cyclophosphamide when compared to the corn oil control; this decrease, however, was not statistically significant ($P > 0.15$). As in the male, the content of cytochrome P-450 in female liver was increased 1.4-fold by β-naphthoflavone pretreatment.

Activation of cyclophosphamide to mutagenic metabolites by the 9000 *g* supernatant fraction from the kidneys of male and female control and phenobarbital-pretreated rats is illustrated in Fig. 4. Enzymatic activation of cyclophosphamide to mutagenic metabolites by control male kidney S9 was significantly higher ($P < 0.05$) than the non-enzymatic mutagenic response (150 μl of S9 fraction/plate). Enzymatic activation by male kidney S9 fraction was approximately one-third of that obtained with liver microsomes from control animals. Since there was no difference between the liver S9 fraction and the liver microsomal fraction with respect to ability to activate cyclophosphamide [10], the decreased activity was not due to the use of the S9 fraction. Phenobarbital pretreatment did not result in a further increase in the enzymatic activation of cyclophosphamide ($P > 0.15$) or in kidney cytochrome P-450 content (Table 1).

Activation of cyclophosphamide to mutagenic metabolites by female kidney S9 fraction was not linear with the amount of S9 fraction, nor was it significantly different from the non-enzymatic mutagenic response to cyclophosphamide. Phenobarbital pretreatment had no effect on renal cytochrome P-450 content of the female rat (Table 1) or on the ability of S9 fractions from female kidneys to activate cyclophosphamide to mutagenic metabolites.

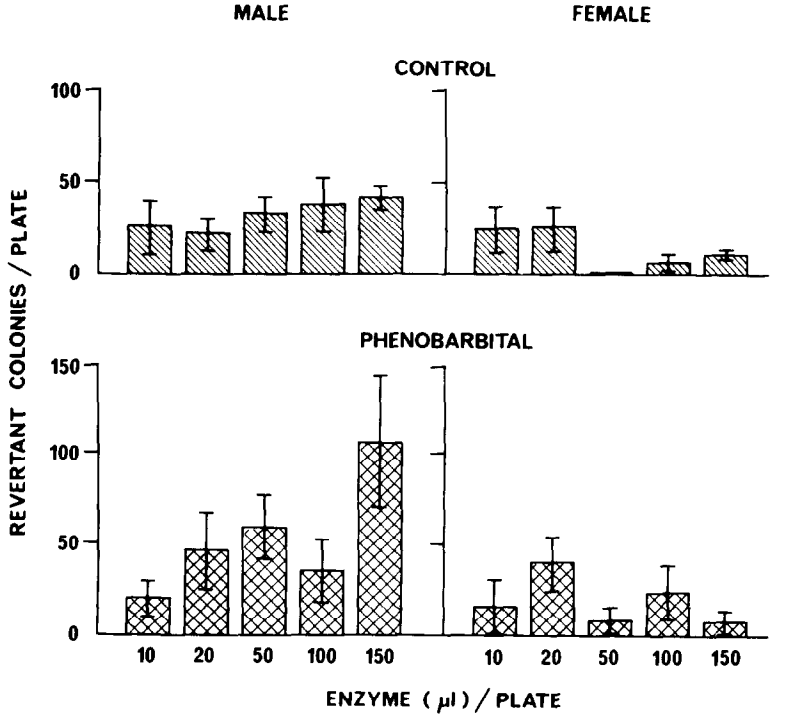

Fig. 4. Comparison of the activation of cyclophosphamide to mutagenic metabolites by untreated and phenobarbital-pretreated male and female rat kidney 9000 *g* supernatant fractions. Experiments were done as described in the legend to Fig. 2. Values are means ± S.E.M. (N = 3).

Activation of cyclophosphamide to mutagenic metabolites by renal S9 fractions from male or female rats pretreated with corn oil did not differ ($P > 0.05$) from the results with cyclophosphamide alone. No sex difference in kidney enzyme was observed in corn oil-treated animals. β-Naphthoflavone pretreatment of either male or female rats did not induce renal cytochrome P-450 (Table 1), nor did it alter the ability of the kidney to activate cyclophosphamide to mutagenic metabolites.

DISCUSSION

Levin *et al.* [26], studying the turnover of cytochrome P-450, found two pools of cytochrome P-450 in rat liver. The half-life of one pool was 7–8 hr and that of the other was 44–48 hr. The ratio of the 'fast' to the 'slow' component was 3.4–4.4 in adult female animals but only 1.9 in adult males. This sex difference in the turnover of cytochrome P-450 found in male and female rat liver is probably a reflection of differences in substrate specificity, e.g. *N*-demethylation of ethylmorphine [11, 14] and activation of cyclophosphamide to alkylating metabolites [2]. Such a sex difference was found in this report with respect to the ability of hepatic microsomes to catalyze the activation of cyclophosphamide to mutagenic metabolites. Microsomes from male liver had a 3-fold greater ability to activate cyclophosphamide to mutagenic metabolites than did microsomes from female rat liver. The consequences of this sex difference in terms of the form(s) of cytochrome P-450 involved in the activation or in the toxicity of cyclophosphamide are not known. This sex difference in hepatic activation of cyclophosphamide was abolished by pretreatment with phenobarbital.

Induction with β-naphthoflavone decreased the abilities of male and female rat liver microsomal fraction to activate cyclophosphamide to mutagenic metabolites. Polycyclic aromatic hydrocarbons (3-methylcholanthrene or β-naphthoflavone) induce more than one form of cytochrome P-450 [27, 28]. The failure of β-naphthoflavone to induce cyclophosphamide activation by hepatic or renal enzyme fractions probably means that aromatic hydrocarbon-induced cytochromes are not involved in this reaction.

Renal activation of cyclophosphamide to mutagenic metabolites was approximately one-third of liver activation in male rats. In female rats, no renal enzymatic activation of cyclophosphamide to mutagenic metabolites was observed. Renal activity was not responsive to induction by either phenobarbital or β-naphthoflavone. Phenobarbital pretreatment has been reported previously to have no effect on renal mono-oxygenase activity or cytochrome P-450 content [29, 30]. However, induction with a polycyclic aromatic hydrocarbon has been reported to increase both renal cytochrome P-450 content and mono-oxygenase activity toward other substrates [27, 28].

In phenobarbital-induced rats, male or female, the predominant site of activation of cyclophosphamide to mutagenic metabolites appears to be the liver. In male rats the kidney probably also contributes to the activation of cyclophosphamide. This contribution could be important in the local production of reactive metabolites *in vivo*. Such metabolites might have a role in the production of cystitis [20, 31] or of bladder cancer associated with the administration of cyclophosphamide. If toxic metabolites of cyclophosphamide are produced by the kidney, selective inhibition of renal mixed function oxidase activity might decrease the incidence of toxic effects without altering the therapeutic efficacy of cyclophosphamide.

In summary, there are tissue and sex differences in the activation of cyclophosphamide to mutagenic metabolites *in vitro* in control animals, and these differences can be compounded by pretreatment with xenobiotics. The *in vivo* significance of these differences in terms of cyclophosphamide toxicity remains to be elucidated.

Acknowledgements—The present study was supported by Grant MA-7078 from the Medical Research Council of Canada. The assistance of Dr. B. Robaire in the statistical analyses of these results is gratefully acknowledged.

REFERENCES

1. G. E. Foley, O. M. Friedman and B. P. Drolet, *Cancer Res.* **21**, 57 (1961).
2. N. E. Sladek, *Cancer Res.* **31**, 901 (1971).
3. N. E. Sladek, *Cancer Res.* **33**, 1150 (1973).
4. J. Ellenberger and G. R. Mohn, *J. Toxic. envir. Hlth* **3**, 637 (1977).
5. W. F. Benedict, A. Banerjee and N. Venkatesan, *Cancer Res.* **38**, 2922 (1978).
6. W. J. Suling, R. F. Struck, C. W. Woolley and W. M. Shannon, *Cancer Treat. Rep.* **62**, 1321 (1978).
7. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **56**, 898 (1974).
8. M. Warner, M. V. La Marca and A. H. Neims, *Drug Metab. Dispos.* **6**, 353 (1978).
9. D. E. Ryan, P. E. Thomas, D. Korzeniouwski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
10. B. F. Hales and R. Jain, *Biochem. Pharmac.* **29**, 256 (1980).
11. J. A. Castro and J. R. Gillette, *Biochem. biophys. Res. Commun.* **28**, 426 (1967).
12. S. El Defrawy El Masry, G. M. Cohen and G. J. Mannering, *Drug Metab. Dispos.* **52**, 267 (1974).
13. R. Kato, *Drug Metab. Rev.* **3**, 1 (1974).
14. D. E. Nerland and G. J. Mannering, *Drug Metab. Dispos.* **6**, 150 (1978).
15. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134**, 76 (1969).
16. F. P. Guengerich and P. S. Mason, *Molec. Pharmac.* **15**, 154 (1979).
17. N. Brock and H-J. Hohorst, *Arzneimittel-Forsch.* **13**, 1021 (1963).
18. T. Kondo and H. Muragishi, *Gann* **61**, 145 (1970).
19. D. L. Hill, W. R. Laster, Jr. and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
20. N. Brock, J. Stekar, J. Pohl, U. Niemeyer and G. Scheffler, *Arzneimittel-Forsch.* **29**, 659 (1979).
21. S. Levine and R. Sowinski, *Invest. Urol.* **13**, 196 (1975).
22. B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.* **31**, 347 (1975).
23. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. G. W. Snedecor and W. G. Cochran, in *Statistical Methods*, 6th Edn. pp. 120–307. Iowa State University Press, Ames, IA (1967).

26. W. Levin, D. Ryan, R. Kuntzman and A. H. Conney, *Molec. Pharmac.* **11**, 190 (1975).
27. S. S. Thorgeirsson, S. A. Atlas, A. R. Boobis and J. S. Felton, *Biochem. Pharmac.* **28**, 217 (1979).
28. A. R. Boobis, D. W. Nebert and J. S. Felton, *Molec. Pharmac.* **13**, 259 (1977).
29. B. G. Lake, R. Hopkins, J. Chadraborty, J. W. Bridges and D. V. W. Parke, *Drug Metab. Dispos.* **1**, 342 (1973).
30. W. M. Kluwe, K. M. McCormack and J. B. Hook, *J. Pharmac. exp. Ther.* **207**, 566 (1978).
31. P. J. Cox, *Biochem. Pharmac.* **28**, 2045 (1979).