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EFFECT OF PHENOBARBITAL OR PREGNENOLONE-16α-CARBONITRILE (PCN) PRETREATMENT ON ACUTE CARBON TETRACHLORIDE HEPATOTOXICITY IN RATS

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Abstract— In rats, CCl_4 hepatotoxicity (reflected by augmented serum glutamic-pyruvic transaminase activity and hepatic triglyceride content) was diminished by pregnenolone-16α-carbonitrile (PCN) and markedly increased by phenobarbital. PCN, like phenobarbital, caused smooth-surfaced endoplasmic reticulum proliferation in hepatocytes, and enhanced hexobarbital oxidation, ethylmorphine *N*-demethylation, cytochrome P-450 and NADPH-cytochrome c reductase activity. Contrary to earlier views, it is concluded that the increase in these parameters is not a prerequisite for augmented CCl_4 toxicity. Perhaps the cytochrome P-450 induced by the steroid and barbiturate has different catalytic properties, which are responsible for variations in the response to CCl_4.

NUMEROUS recent investigations[1–7] have dealt with the molecular basis of CCl_4 hepatotoxicity. Yet, the exact site where it is converted into a toxic metabolite remains unknown. Some authors[1–7] have suggested that cleavage of CCl_4 (possibly into $\cdot CCl_3$) occurs in the hepatocytic endoplasmic reticulum, and that free radicals attack the lipoidal and proteinic elements of this organelle. According to Recknagel and Ghoshal,[8] the initial peroxidative breakdown of these membranes is the key event which triggers subsequent alterations characteristic of CCl_4 intoxication.

Phenobarbital pretreatment enhances hepatic microsomal drug-metabolizing enzyme activity, produces marked proliferation of the smooth-surfaced endoplasmic reticulum (SER) in hepatocytes,[9,10] and augments CCl_4 toxicity.[11–13] Drug-metabolizing enzymes are also induced by pregnenolone-16α-carbonitrile (PCN),[14] but the enzyme system has substrate specificities that are different from those observed after phenobarbital[15,16] or 3-methylcholanthrene (3-MC) administration.[17] In this study, we compared the effects of hepatic microsomal enzyme induction by phenobarbital and PCN on acute CCl_4 hepatotoxicity.

MATERIALS AND METHODS

Female Sprague–Dawley rats (Canadian Breeding Farms & Laboratories Ltd., St. Constant, Quebec), weighing 110–170 g, were maintained *ad lib.* on Purina Labora-

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tory Chow (Ralston Purina Co. of Canada) and tap water. In the first experiment (see Tables 1 and 2), Na-phenobarbital (B.D.H.) and PCN [3β-hydroxy-20-oxo-5-pregnene-16α-carbonitrile (Upjohn)] were given *per os* at dose levels of 46 and 68 mg/kg of body weight, respectively, in 1 ml water, the latter as a micronized suspension with a trace of Tween 80. Both compounds were administered at 8-hr intervals (twice daily for 2 days, and once on the 3rd day). For comparative purposes, the controls received water.

All animals were sacrificed by decapitation 24 hr after the last phenobarbital or PCN gavage. Their livers were immediately removed, weighed and washed in an ice-cold 1·15% KCl solution. Samples were taken and processed at 0–3°.

After gentle homogenization of 1 g liver in 3 vol. of isotonic KCl containing 0·2 M Tris-HCl (pH 7·4), the samples were centrifuged at 9000 *g* for 20 min. The resulting supernatant fractions were then centrifuged at 105,000 *g* for 60 min. The microsomal pellets obtained were washed and recentrifuged at 105,000 *g* for 30 min. Microsomal suspensions were prepared in 1·15% KCl 0·02 M Tris-HCl (pH 7·4) by gentle homogenization.

The amount of microsomal protein was estimated by the method of Lowry *et al.*,[18] using crystalline bovine serum albumin as a standard. Cytochrome P-450 content was determined by the Omura–Sato[19] technique, employing an extinction coefficient of 91 cm^{-1} mM^{-1}. NADPH-cytochrome c reductase activity was measured according to a method described by Holtzman *et al.*[20] Ethylmorphine *N*-demethylation was assessed[21] by using the Nash[22] reaction to determine the amount of formaldehyde formed. Hexobarbital oxidation was measured according to the method of Brodie *et al.*,[23] as modified by Remmer.[24]

The incubation medium for the enzyme assays consisted of: Tris-HCl buffer, pH 7·4, 0·1 M; $MgCl_2$, 5 mM; glucose 6-phosphate, 5 mM; glucose 6-phosphate dehydrogenase, 0·7 unit/ml; NADP, 0·4 mM; microsomal protein, 2 mg/ml; ethylmorphine, 1 mM; hexobarbital, 0·4 mM. For ethylmorphine and hexobarbital, the duration of incubation was 10 and 20 min respectively.

For electron microscopy, small samples of liver tissue were placed in Millonig's osmium fixative, where they were minced into tiny cubes and kept for 1 hr at 4°. These specimens were then dehydrated in graded ethanol and embedded in Epon resin. For lobular localization, sections, 0·5 μm thick, were cut on a Porter Blum MT-2 microtome, stained with toluidine blue, and examined with a light microscope. Ultrathin sections (approximately 50 nm) were cut, stained with uranyl acetate and Reynolds' lead citrate, and examined with a Carl Zeiss EM 9A electron microscope.

In the second experiment (see Table 3), comparable groups of rats were challenged with CCl_4 [2·5 ml/kg (Fisher) of a 50 per cent solution, in corn oil, *per os*] 24 hr after the last phenobarbital or PCN gavage. These animals were killed 24 hr later, under ether anesthesia, by withdrawal of blood through the abdominal aorta. As an index of CCl_4 hepatotoxicity, serum glutamic-pyruvic transaminase (SGPT) activity was determined with a Dade reagent kit, and the hepatic triglyceride level measured according to a modification of the van Handel–Silversmith method.[25] The differences were statistically evaluated by Student's *t*-test. For light microscopy, liver tissue was fixed in alcohol–formol or neutral formalin and embedded in paraffin. Sections, 4 μm thick, were cut and stained with hematoxylin–phloxine. Frozen sections were stained with Oil red O.

TABLE. 1. EFFECT OF PHENOBARBITAL AND PCN ON HEPATIC WEIGHT, MICROSOMAL PROTEIN, CYTOCHROME P-450 AND NADPH-CYTOCHROME c REDUCTASE*

Pretreatment	Liver weight (g/100 g body weight)	Microsomal protein recovered (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Increase (%)	NADPH-cytochrome c reductase (nmoles reduced/ mg protein/min)	Increase (%)
None	4·32 ± 0·03	18·38 ± 0·81	0·74 ± 0·02		188 ± 16	
Phenobarbital	5·62 ± 0·09†	22·73 ± 0·45‡	1·43 ± 0·06†	95	283 ± 4§	50
PCN	5·20 ± 0·17§	21·20 ± 0·56‡	1·18 ± 0·03†	60	316 ± 3†	70

* Phenobarbital and PCN were administered on an equimolecular basis (0·2 nmole/kg body wt, p.o., twice daily for 2 days, and once on the third day). All rats were decapitated 24 hr after the last gavage. The values represent the mean ± S.E. of four rats per group.

† $P < 0·001$ compared with group 1.

‡ $P < 0·05$ compared with group 1.

§ $P < 0·01$ compared with group 1.

TABLE 2. EFFECT OF PHENOBARBITAL AND PCN ON HEPATIC MICROSOMAL HEXOBARBITAL OXIDATION AND ETHYLMORPHINE *N*-DEMETHYLATION*

Pretreatment	Hexobarbital oxidation (nmoles disappeared/ mg protein/min)	Increase (%)	Ethylmorphine *N*-demethylation (nmoles HCHO formed/ mg protein/min)	Increase (%)
None	1·16 ± 0·03		3·00 ± 0·06	
Phenobarbital	5·17 ± 0·15†	345	13·38 ± 0·10†	345
PCN	1·74 ± 0·10†	50	19·57 ± 0·34†	550

* Phenobarbital and PCN were given on an equimolecular basis (0·2 nmole/kg body wt, p.o., twice daily for 2 days, and once on the 3rd day). All animals were decapitated 24 hr after the last gavage. The values represent the mean ± S.E. of four rats per group.

† $P < 0·001$ compared with group 1.

RESULTS

As seen in Table 1, phenobarbital and PCN significantly increased liver weight, microsomal protein and cytochrome P-450 content, as well as NADPH-cytochrome c reductase activity. Table 2 shows that phenobarbital greatly stimulated hexobarbital oxidation, whereas PCN markedly augmented ethylmorphine *N*-demethylation. Both enzyme inducers caused pronounced SER proliferation in hepatocytes. This finding has been extensively reported elsewhere;[9,10,12,26–28] hence, further description would be redundant.

In the hepatotoxicity studies, the SGPT values and the triglyceride content of the liver were markedly increased 24 hr after CCl_4 treatment (Table 3). These changes were further enhanced by phenobarbital. In contrast, the SGPT levels and the hepatic triglycerides were significantly decreased by PCN as compared with CCl_4-treated controls.

Histologically, CCl_4 produced moderate centrilobular necrosis of the liver. The cells in this zone showed hydropic changes and inflammatory infiltration or mild vacuolization of the cytoplasm. The Oil red O-stained sections exhibited fatty infiltration of the central and periportal areas. In phenobarbital-pretreated rats, liver necrosis was accentuated; cellular vacuolization virtually encompassed the entire hepatic parenchyma. Centrilobular necrosis was limited to small areas in animals

TABLE 3. EFFECT OF PHENOBARBITAL OR PCN PRETREATMENT ON SGPT ACTIVITY, LIVER WEIGHT AND TRIGLYCERIDE CONTENT IN CCl_4-INTOXICATED RATS*

Group	Treatment	SGPT (units/ml plasma ± S.E.)	Liver wt (g/100 g body wt ± S.E.)	Triglycerides (mg/g wet liver ± S.E.)
1	None	44 ± 3	4·8 ± 0·1	7·4 ± 0·7
2	CCl_4	318 ± 49†	5·7 ± 0·1‡	31·0 ± 3·6†
3	CCl_4 + phenobarbital	1086 ± 92§	7·4 ± 0·2¶	102·0 ± 1·9¶
4	CCl_4 + PCN	163 ± 28§	6·6 ± 0·2¶	18·0 ± 1·9¶

* Phenobarbital and PCN were given on an equimolecular basis (0·2 nmole/kg body wt), p.o., twice daily on days 1 and 2, and once on day 3. Twenty-four hr after the last gavage, all rats were given CCl_4 (1·25 ml/kg, p.o.). Blood and liver samples were taken 24 hr later.

† $P < 0·001$ compared with group 1.

‡ $P < 0·01$ compared with group 1.

§ $P < 0·001$ compared with group 2.

¶ $P < 0·01$ compared with group 2.

given PCN. Cells with "ballooning" or cytoplasmic vacuolization were less numerous than in CCl_4-treated controls. The periportal zones were well preserved. Fatty infiltration was decreased.

DISCUSSION

It is generally believed that CCl_4-induced hepatic damage is related to the formation of a toxic metabolite (possibly $\cdot CCl_3$) somewhere along the microsomal electron transfer system of the hepatocyte.[2,3,5] The drug-metabolizing enzyme system, particularly NADPH-cytochrome c reductase[2] and cytochrome P-450,[11,29] may be involved in the conversion of CCl_4. This view is supported by the finding that phenobarbital pretreatment (which increases cytochrome P-450 and NADPH-cytochrome c reductase) greatly enhances susceptibility to CCl_4.[11-13] Aggravation of toxicity has also been associated with SER proliferation produced by the barbiturate in hepatocytes.[30]

However, it appears that the drug-metabolizing enzyme system rather than SER proliferation *per se* is the eliciting factor. Other experiments[31,32] have revealed that CCl_4-induced hepatic damage is not directly related to the levels of drug-metabolizing enzymes, cytochrome P-450 or NADPH-cytochrome c reductase. In rats, pretreatment with 3-MC or benzpyrene (BP) causes an increase of drug-metabolizing enzymes and cytochrome P-448, with no change in cytochrome c reductase activity; yet, 3-MC prevents,[31,33] whereas BP enhances[32] CCl_4 hepatotoxicity. Both polycyclic hydrocarbons elicit little or no SER proliferation in hepatocytes.[27,34] Experiments in which drug-metabolizing enzyme activity was inhibited by dietary means (e.g. protein-free diets, starvation) or drug treatment (e.g. SKF 525-A) did not always result in decreased CCl_4 hepatotoxicity.[13,35] It is possible that, while cleavage of CCl_4 and drug detoxication (through active P-450 participation) are both handled by the same transfer system, the sites and pathways of the two are different. Activation may occur at the initial stages of the microsomal electron transport chain, although the exact site is not yet known.[35] Perhaps the protection offered by 3-MC is due to altered pathways of CCl_4 metabolism, causing a decrease of toxic metabolite formation.[33] A similar explanation has been put forward for the prevention of CCl_4 toxicity by the synthetic antioxidant, ethoxyquin, which also induces drug-metabolizing enzymes.[36]

The present studies indicate that neither SER proliferation nor increased drug-metabolizing enzyme activity, particularly of cytochrome P-450 and NADPH-cytochrome c reductase, is a prerequisite for enhanced susceptibility to CCl_4. PCN augments all these parameters but, unlike phenobarbital, it decreases hepatotoxicity. Most probably, the steroid and the barbiturate induce cytochrome P-450 with different catalytic properties,[17] this, in turn, being responsible for the variations in substrate specificities including the response to CCl_4.

Studies *in vivo*[37] have shown that PCN pretreatment prevents diverse intoxications. The steroid protects against hepatic lipidosis caused by $CeCl_3$[37] or beloxamide,[38] which do not seem to undergo metabolism in liver microsomes. PCN, like other steroids, may stabilize cell membranes and render the hepatocyte less susceptible to the effects of various toxins.

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REFERENCES

1. T. C. BUTLER, *J. Pharmac. exp. Ther.* **134,** 311 (1961).
2. T. F. SLATER, *Nature, Lond.* **209,** 36 (1966).
3. R. O. RECKNAGEL, *Pharmac. Rev.* **19,** 145 (1967).
4. K. S. RAO and R. O. RECKNAGEL, *Expl molec. Path.* **10,** 219 (1969).
5. E. A. SMUCKLER and M. ARCASOY, *Int. Rev. exp. Path.* **7,** 305 (1969).
6. E. S. REYNOLDS and H. J. REE, *Lab. Invest.* **25,** 269 (1971).
7. K. S. RAO and R. O. RECKNAGEL, *Expl. molec. Path.* **12,** 324 (1970).
8. R. O. RECKNAGEL and A. K. GHOSHAL, *Lab. Invest.* **15,** 132 (1966).
9. S. ORRENIUS, J. L. E. ERICSSON and L. ERNSTER, *J. Cell Biol.* **25,** 627 (1965).
10. H. REMMER and H. J. MERKER, *Ann. N.Y. Acad. Sci.* **123,** 79 (1965).
11. R. C. GARNER and A. E. M. MCLEAN, *Biochem. Pharmac.* **18,** 645 (1969).
12. R. J. STENGER, R. A. MILLER and J. N. WILLIAMSON, *Expl molec. Path.* **13,** 242 (1970).
13. B. TUCHWEBER and K. KOVACS, *Arch. Tox.* **27,** 159 (1971).
14. B. SOLYMOSS, J. WERRINGLOER and S. TOTH, *Steroids* **17,** 427 (1971).
15. J. WERRINGLOER, *Fedn Proc.* **31,** 641 (1972).
16. J. WERRINGLOER, *Proc. Fifth Int. Cong. Pharmacology*, p. 250, No. 1500 (1972).
17. A. Y. H. LU, A. SOMOGYI, S. WEST, R. KUNTZMAN and A. H. CONNEY, *Archs Biochem. Biophys.* **152,** 457 (1972).
18. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
19. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
20. J. L. HOLTZMAN, T. E. GRAM, P. L. GIGON and J. R. GILLETTE, *Biochem. J.* **110,** 407 (1968).
21. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3,** 113 (1967).
22. T. NASH, *Biochem. J.* **55,** 416 (1953).
23. B. B. BRODIE, J. J. BURNS, L. C. MARK, P. A. LIEF, E. BERNSTEIN and E. M. PAPPER, *J. Pharmac. exp. Ther.* **109,** 26 (1953).
24. H. REMMER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **233,** 173 (1958).
25. W. M. BUTLER, JR., H. M. MALING, M. G. HORNING and B. B. BRODIE, *J. Lipid Res.* **2,** 95 (1961).
26. B. D. GARG, J. A. BLASCHECK and K. KOVACS, *Tohoku J. exp. Med.* **104,** 205 (1971).
27. B. D. GARG, K. KOVACS, J. A. BLASCHECK and H. SELYE, *J. Pharm. Pharmac.* **22,** 872 (1970).
28. B. TUCHWEBER, B. SOLYMOSS, J. D. KHANDEKAR, K. KOVACS, B. D. GARG, G. ZSIGMOND and R. DOBARDZIC, *Expl molec. Path.* **17,** 281 (1972).
29. J. A. CASTRO, M. I. DIAZ GOMEZ, E. C. FERREYRA, C. R. DE CASTRO, N. DE D'ACOSTA and O. M. FENOS, *Biochem. biophys. Res. Commun.* **47,** 315 (1972).
30. R. J. STENGER and E. A. JOHNSON, *Expl molec. Path.* **14,** 220 (1971).
31. K. A. SUAREZ, G. P. CARLSON, G. C. FULLER and N. FAUSTO, *Toxic. appl. Pharmac.* **23,** 171 (1972).
32. C. S. PITCHUMONI, R. J. STENGER, W. S. ROSENTHAL and E. A. JOHNSON, *J. Pharmac. exp. Ther.* **181,** 227 (1972).
33. B. STRIPP, M. E. HAMRICK and J. R. GILLETTE, *Biochem. Pharmac.* **21,** 745 (1972).
34. J. R. FOUTS and L. A. ROGERS, *J. Pharmac. exp. Ther.* **147,** 112 (1965).
35. P. CHOPRA, S. ROY, V. RAMALINGASWAMI and N. C. NAYAK, *Lab. Invest.* **26,** 716 (1972).
36. M. A. CAWTHORNE, E. D. PALMER and J. GREEN, *Biochem. Pharmac.* **22,** 783 (1973).
37. H. SELYE, *Hormones and Resistance*, p. 216. Springer, Heidelberg (1971).
38. H. SELYE and F. LEFEBVRE, *Archs Anat. Path.* **19,** 127 (1971).