Biochemical Pharmacology, Vol. 18, pp. 645–650. Pergamon Press. 1969. Printed in Great Britain

INCREASED SUSCEPTIBILITY TO CARBON TETRACHLORIDE POISONING IN THE RAT AFTER PRETREATMENT WITH ORAL PHENOBARBITONE

R. C. GARNER and A. E. M. MCLEAN

Department of Experimental Pathology, University College Hospital Medical School, London, W.C.1

(*Received* 22 *July* 1968; *accepted* 11 *October* 1968)

Abstract—Pretreatment of rats with phenobarbitone, dissolved in the drinking water, increases their susceptibility to CCl_4 poisoning. The median lethal dose (LD50) falls from 3·6 ml CCl_4/kg in control animals to 0·5 ml/kg in phenobarbitone pretreated animals. 0·25 ml/kg of CCl_4 administered orally produces severe liver damage in phenobarbitone pretreated animals as shown by high liver fat and water content, and high plasma isocitrate dehydrogenase and bilirubin levels. This dose of CCl_4 produces only slight damage in control animals. Control animals given 2·5 ml/kg CCl_4 have a similar degree of liver damage to that found in phenobarbitone treated animals given 0·25 ml/kg CCl_4. Both groups metabolise about 12 mg/kg of the administered CCl_4 to CO_2, in 6 hr.

Phenobarbitone pretreatment increases metabolism of CCl_4 to CO_2 both *in vivo* and *in vitro*.

Comparison of CCl_4 levels in the blood and liver of pretreated and control animals suggest that the toxic effects cannot be related to the action of unaltered CCl_4, and strengthen the view that CCl_4 must be metabolised to cause cell damage. The nature of the toxic metabolite remains uncertain.

THE SUSCEPTIBILITY of animals to CCl_4 poisoning is very variable and depends upon both the species and the diet. Susceptibility can be assessed by measuring LD50 and by measuring indices of liver damage after varying doses of CCl_4. Previous work has shown that when rats or sheep are fed on low protein diets they become highly resistant to the lethal and liver damaging effects of CCl_4.[9, 10, 16] Both these species when fed on a low protein diet have a greatly reduced ability to "detoxicate" drugs, such as barbiturates, and to carry out those reactions which are catalysed by the liver microsomal hydroxylation system. The ability of the protein depleted animals to metabolise CCl_4 to carbon dioxide is depressed, both *in vivo* and in liver microsome preparations. On the administration of either phenobarbitone or dicophane (DDT) by injection, the activity of the microsomal hydroxylating system is restored and the animals become susceptible to the poison once more.[10, 15]

Liver damage caused by CCl_4 can be assessed by measuring a number of parameters. The ones that we have found most useful are the increase in liver fat and water content, and plasma isocitrate dehydrogenase and bilirubin levels. Previous work has described the changes in these parameters in normal, in protein depleted, and in vitamin E deficient animals, after oral administration of CCl_4.[7, 10]

Phenobarbitone, administered by injection, causes a massive induction of microsomal hydroxylating enzyme activity.[4, 13] Recently we have described a more convenient and equally effective method of administration in which the phenobarbitone

is given as a 1 mg/ml solution in the drinking water.[11, 12] Phenobarbitone pretreatment lasting for 2 weeks or more, before CCl_4 dosage has been used throughout the experiments described in this paper.

The present study was carried out to see whether the susceptibility to CCl_4 poisoning of rats fed normal diets was increased by pretreatment with phenobarbitone and if this could be simply related to the amount of CCl_4 metabolised.

MATERIALS AND METHODS

Rats. Male Carworth rats weighing between 100–120 g were fed *ad libitum* on a stock cube diet (diet 41B of Bruce & Parkes)[1] in cages with mesh floors to limit coprophagy. Sodium phenobarbitone (British Drug Houses, Poole, Dorset) (1 mg/ml) in distilled water was administered as drinking water for at least 14 days. Unless stated otherwise, all rats were fasted and tap water substituted for the phenobarbitone solution 18 hr before the CCl_4 experiments were started.

Dosing with CCl_4. CCl_4 (Analar Grade, British Drug Houses), was given orally as a mixture with light liquid paraffin (sp.gr. 0·83–0·87; B.D.H.). When a dose of 0·25 ml/kg CCl_4 was required, this was always given as a 1 + 7 (v/v) mixture. The rats were lightly anaesthetised with ether and the CCl_4 was given intragastrically with a fine, soft-rubber catheter. The dose was always given between 10 a.m. and 11 a.m. Rats were killed by exsanguination from the carotid artery under ether anaesthesia.

Determination of LD50. LD50 values were determined and calculated on four groups of four rats as described by Weil[17] and McLean and McLean.[10]

Chemical Determination. Plasma isocitrate dehydrogenase (ICD) and bilirubin levels, liver fat and water content were measured and calculated as previously described.[10] The use of log values for plasma enzyme activities is also discussed by Heath.[6]

Metabolism of $^{14}CCl_4$ in vivo. $^{14}CCl_4$ was purchased from the Radiochemical Centre, Amersham, Bucks. $^{14}CO_2$ was removed by equilibration against carrier carbonate. Rats were given 0·25 ml/kg of $^{14}CCl_4$ (sp. act. 100 μc/ml) as a 1 + 7 (v/v) dilution with liquid parafin, intragastrically. The rat was placed in a metabolism cage (Metabowl. Jencons Ltd. Hemel Hempstead, Herts) and air was drawn through the cage at a flow rate of 600 ml/min. Expired $^{14}CO_2$ and $^{14}CCl_4$ were absorbed and measured as previously described.[15]

Metabolism of $^{14}CCl_4$ in vitro. Post mitochondrial supernatant was prepared from rat livers as follows. A 2 g piece of liver was homogenised with 18 ml of 0·15 M KCl in a blender (Ultra–Turrax, Janke and Kunkel K.G., Staufen im Breisgau, Germany) for 5 sec. The homogenate was then centrifuged for 10 min at 9000 *g* at 4° in a Sorvall RC2-B refrigerated centrifuge to remove cell debris, mitochondria, nuclei and erythrocytes. The supernatant was decanted and 1 ml samples added to a stock mixture in a Warburg flask. The procedure described by Seawright and McLean,[15] was then followed except that nicotinamide was omitted from the stock mixture.

$^{14}CCl_4$ concentrations in liver and blood. These were measured using the method of Dawkins.[2]

RESULTS

The LD50 for CCl_4 was 0·5 ml/kg in rats pretreated with phenobarbitone compared with 3·6 ml/kg for control rats (Table 1). Removing phenobarbitone 18 hr before dosing with CCl_4 instead of at the time of dosing did not alter the lethal effect.

Table 2 shows that treatment with phenobarbitone causes an increase in liver fat content, in the absence of CCl_4. In stock rats a small dose of CCl_4 (0·25 ml/kg) causes an increase in plasma isocitrate dehydrogenase activity, no increase in plasma bilirubin, but a significant increase in liver water and fat content ($P < 0·002$ by Student's *t* test) in comparison with normal controls. In phenobarbitone pretreated rats, both fed and fasted, this small dose of CCl_4 caused large increases in the four indices of liver damage that we used.

When we measured CCl_4 metabolism *in vitro* we found that 3 mM CCl_4 was converted to carbon dioxide at a rate of 4·3 ± 0·53 μmole/g of liver/hr using the post mitochondrial supernatant. 10 mM CCl_4 gave a rate of 6·5 ± 1·24 μmole/g of liver/hr (means ± S.D.).

Figure 1 shows that phenobarbitone pretreatment caused a marked stimulation of the metabolism of $^{14}CCl_4$ to $^{14}CO_2$ *in vivo*. Expiration of unaltered $^{14}CCl_4$ was not affected.

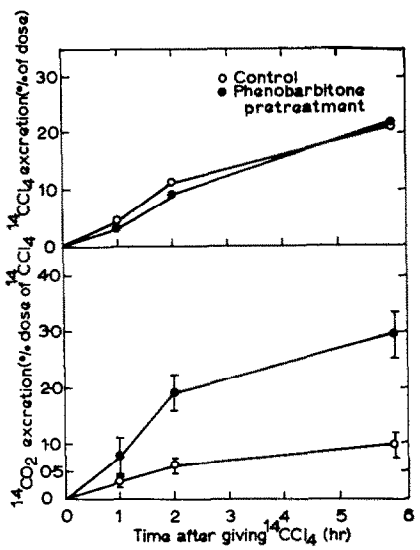

FIG. 1. The effect of phenobarbitone pretreatment on expiration of $^{14}CCl_4$ and $^{14}CO_2$ after an oral dose of $^{14}CCl_4$ 0·25 ml/kg.

Table 3 shows the liver and blood concentrations of CCl_4, 1 and 3 hr after dosing. In animals given 2·5 ml/kg the concentration of CCl_4 found in the liver was highly variable, but was always large in comparison with the concentration found in animals given 0·25 ml/kg.

DISCUSSION

In this paper we have shown in rats fed stock diets that pretreatment with phenobarbitone in the drinking water, greatly increases the sensitivity to the lethal and hepatoxic effects of CCl_4.

The magnitude of this increased sensitivity has been demonstrated in two ways. First the LD50 for CCl_4 is lowered by a factor of about seven by phenobarbitone pretreatment. Secondly, the indices of liver damage are similar in pretreated rats given 0·25 ml/kg CCl_4, and in control rats given a tenfold greater dose.

TABLE 1. ENHANCEMENT OF THE LETHAL EFFECTS OF CCl_4 IN RATS PRETREATED WITH PHENOBARBITONE

Diet	LD50 (ml/kg)	95% confidence limits
Cube diet	3·6	2·7–4·9
Cube diet + phenobarbitone	0·5	0·4–0·6

Male rats were pretreated with phenobarbitone in the drinking water until dosing with CCl_4, when tap water was substituted. Food was allowed at all times. CCl_4 dosage and calculation of LD50 are described in the Methods section. The rats weighed 200–250 g at dosing and deaths were recorded for 1 week after dosing.

TABLE 2. THE EFFECT OF PRETREATMENT WITH PHENOBARBITONE ON INDICES OF LIVER DAMAGE PRODUCED BY ORAL CCl_4 (0·25 ml/kg AND 2·5 ml/kg)

Diet	CCl_4 dose ml/kg	No. of animals	Plasma isocitrate dehydrogenase activity (log mμM/ml/min)	Plasma concentration of bilirubin (mg/100 ml)	Liver water content (g/g fat free dry wt.)	Liver fat content (mg/g fat free dry wt.)
Cube diet (fasted)	Nil	4	0·28 ± 0·15	0·11 ± 0·03	2·41 ± 0·06	47 ± 13
Cube diet + phenobarbitone (fasted)	Nil	7	0·56 ± 0·17	0·20 ± 0·11	2·66 ± 0·21	160 ± 31
Cube diet (fasted)	0·25	5	1·07 ± 0·30	0·14 ± 0·06	2·80 ± 0·15	150 ± 6
Cube diet + phenobarbitone (fasted)	0·25	8	2·98 ± 0·24	2·24 ± 0·37	3·73 ± 0·17	286 ± 78
Cube diet + phenobarbitone (fed)	0·25	4	2·40 ± 0·25	0·94 ± 0·34	3·32 ± 0·17	293 ± 13
Cube diet (fasted)	2·5	5	2·72 ± 0·11	1·39 ± 0·33	3·42 ± 0·26	377 ± 136*

Results are expressed as mean ± S.D. Rats were pretreated with or without phenobarbitone in the drinking water for 14 days. Sixteen hours before CCl_4 administration tap water was substituted, and food was removed from the fasted groups. Five hours after administration of CCl_4 food was returned to the fasted groups. Controls were intubated with liquid paraffin alone. Twenty-four hours after CCl_4 dosage the rats were killed and blood and liver samples taken.

* The results for the cube diet fasted group given 2·5 ml/kg CCl_4 were taken from Ref. 7 for comparison.

TABLE 3. BLOOD AND LIVER CONCENTRATIONS OF CCl_4 AFTER A DOSE OF 0·25 ml/kg.

Diet	CCl_4 dose (ml/kg)	Time after dose (hr)	No. of animals	CCl_4 in blood (μg/ml)	CCl_4 in liver (μg/g/wet wt.)
Cube diet	0·25	1	3	4·9 ± 2·7	45 ± 13
	0·25	3	3	4·8 ± 1·6	16 ± 7
Cube diet + phenobarbitone	0·25	1	3	4·5 ± 2·5	27 ± 3
	0·25	3	3	3·9 ± 0·6	42 ± 7
Cube diet	2·5	3	5	38 ± 19	385 ± 283 *

Results are expressed as mean ± S.D.

Rats which had been pretreated with phenobarbitone were fasted and phenobarbitone removed 16 hr before oral dosing with $^{14}CCl_4$ (0·25 ml/kg., activity 17 × 10^6dpm/ml). The rats were killed 1 and 3 hr after dosing and analyses performed as described in the Methods section.

* The results for the group given 2·5 ml/kg are taken from Ref. 15 for comparison.

CCl_4 levels in the blood, and the rate of expiration of unaltered CCl_4 are not affected by phenobarbitone pretreatment and cannot explain the increased sensitivity. However, the rate of metabolism of CCl_4 to CO_2 *in vivo* is increased by phenobarbitone pretreatment.

The amount of CCl_4 metabolised to CO_2 is approximately 12 mg/kg in the first 6 hr after dosing both in pretreated animals given 0·25 ml/kg, and control animals given 2·5 ml/kg.[15] The similarity between the indices of liver damage and conversion of CCl_4 to CO_2 in these two groups, whose CCl_4 dosage differs by a factor of ten suggests a close link between CCl_4 metabolism and liver damage. The nature of the toxic metabolite is uncertain, but a $CCl^{\bullet}_3$ free radical has been suggested as the toxic agent.[14, 18]

There is a large variability in the amounts of CCl_4 found in the liver. However, the differences between groups are still large in comparison with the variation inside any one group. Three hours after giving 0·25 ml CCl_4/kg controls have 16 μg/g of CCl_4 in the liver, while phenobarbitone pretreated animals have 42 μg/g. Control and protein depleted animals given the larger dose of 2·5 ml/kg have far higher concentrations of CCl_4 in the liver 3 hr after dosing (385 and 575 μg/g, respectively).[15] The amount of liver damage produced in these various circumstances is so far removed from being proportional to the CCl_4 concentration that it becomes difficult to believe that unaltered CCl_4 can be responsible for liver damage.[3]

CCl_4 metabolism *in vitro* has been found to be similar to most other microsomal hydroxylation reactions in the requirements for cofactors, and the response to diet.[15] Many substrates have been shown to cause changes in the optical absorption spectra of microsomes. The spectral changes produced by addition of CCl_4 to microsomes suggest a close attachment of CCl_4 to cytochrome P450, which plays a central role in the microsomal hydroxylation reactions.[8]

Oral phenobarbitone causes an increase in microsomal cytochrome P450 of about 6-fold after 14 days pretreatment.[12]

In the present study we have found that liver preparations from phenobarbitone pretreated rats metabolised 4·3 μmole CCl_4/g of liver/hr in comparison with 1·1 μmole/g of liver/hr for liver from control animals.[15] These lines of evidence converge to convince us that CCl_4 is metabolised by the microsomal hydroxylation system centered around cytochrome P450.

The indices of liver damage that we have used illustrate the difficulties which attend attempts at chemical measures of cell damage. Phenobarbitone treatment does not cause cell necrosis.[5] It does cause an increase in liver fat content which is similar to that produced by a small dose of CCl_4. However, the increase in plasma isocitric dehydrogenase levels still distinguishes the injury due to CCl_4 from the hypertrophic changes produced by phenobarbitone. In the end the qualitative distinctions have to be made histologically and the chemical changes can then be used for quantitative measurements.

In ranging from protein depleted, to stock, and to stock plus phenobarbitone treated animals, the toxicity of CCl_4 varies by a factor of about thirty. The toxicity of other compounds may well be affected in a similar way, and these factors should be kept in mind when assessing the toxicity of drugs and new compounds.

Acknowledgements—This work was supported by grants from the Science Research Council and U.S. Public Health Service.

REFERENCES

1. H. M. BRUCE and A. S. PARKES, *J. anim. Tech. Ass.* **7**, 54 (1956).
2. M. J. R. DAWKINS, *J. Path. Bact.* **85**, 189 (1963).
3. J. V. DINGELL and M. HEIMBERG, *Biochem. Pharmac.* **17**, 1269 (1968).
4. L. ERNSTER and S. ORRENIUS, *Fedn Proc.* **24**, 1190 (1965).
5. J. R. FOUTS and L. A. ROGERS, *J. Pharmac.* **147**, 112 (1965).
6. D. F. HEATH, *Nature, Lond.* **213**, 1159 (1967).
7. A. E. M. MCLEAN, *Br. J. Exp. Path.* **48**, 632 (1967).
8. A. E. M. MCLEAN, *Biochem. Pharmac.* **16**, 2030 (1967).
9. A. E. M. MCLEAN and E. K. MCLEAN, *Biochem. J.* **97**, 31P (1965).
10. A. E. M. MCLEAN and E. K. MCLEAN, *Biochem. J.* **100**, 564 (1966).
11. W. J. MARSHALL and A. E. M. MCLEAN, *Biochem. J.* **107**, 15P (1968).
12. W. J. MARSHALL and A. E. M. MCLEAN, *Biochem. Pharmac.* **18**, 153 (1969).
13. H. REMMER, *Arch. Exp. Path. Pharmak.* **235**, 279 (1959).
14. E. S. REYNOLDS, *Lab. Invest.* **16**, 591 (1967).
15. A. A. SEAWRIGHT and A. E. M. MCLEAN, *Biochem. J.* **105**, 1055 (1967).
16. A. A. SEAWRIGHT and A. E. M. MCLEAN, *Aust. Vet. J.* **43**, 354 (1967).
17. C. S. WEIL, *Biometrics* **8**, 249 (1952).
18. Z. T. WIRTSCHAFTER and M. W. CRONYN, *Arch. Environ. Hlth.* **9**, 180 (1964).