Biochemical Pharmacology, Vol. 22, pp. 783–788. Pergamon Press, 1973. Printed in Great Britain.

EFFECT OF 6-ETHOXY 2,2,4-TRIMETHYL-1,2-DIHYDROQUINOLINE (ETHOXYQUIN) ON CARBON TETRACHLORIDE METABOLISM IN THE RAT

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(*Received* 1 *July* 1972; *accepted* 25 *October* 1972)

Abstract—Prior administration of ethoxyquin to rats partially prevented the hepatotoxic effects of CCl_4. In rats, given an oral dose of $^{14}CCl_4$ (2 ml/kg), ethoxyquin pretreatment enhanced the overall rate of elimination in the expired air of both $^{14}CCl_4$ and $^{14}CO_2$. However, the rate of metabolism of CCl_4 by liver homogenates was not affected by the ethoxyquin pretreatment. The results support the view that ethoxyquin selectivity induces a CCl_4-detoxification pathway.

THE susceptibility of animals to CCl_4 poisoning, as assessed by measuring the LD_{50}, is very variable and depends upon a number of factors, including the species and the diet. It has been shown that rats or sheep fed diets low in protein become more resistant to the lethal and damaging effect of CCl_4.[1–3] Rats fed a protein-deficient diet are known to have a low drug-metabolizing enzyme activity.[4] Consequently, the finding that the protective effect of the low-protein diet on CCl_4 lethality could be overcome by treatment of the animals with either phenobarbitone or DDT[2,5,6] (which are inducers of drug metabolizing enzymes[7,8]) led to the suggestion that a close correlation existed between the activity of the hepatic drug-metabolizing enzyme system and the toxicity of CCl_4.[5] Indirect evidence for this suggestion is provided by the finding that new-born rats, which are deficient in drug-metabolizing enzymes, are resistant to the toxic effects of CCl_4.[9,10] Furthermore, adult male rats, which have a higher level of drug-metabolizing enzymes than female rats, are also more susceptible to CCl_4 poisoning.[11]

Examination of the rat liver enzyme system that metabolizes CCl_4 revealed that CO_2 was an end-product[12] and that the enzyme system required NADPH and O_2 and was inhibited by CO.[3] Furthermore, when CCl_4 was added to a microsomal preparation containing cytochrome P-450, a typical type I difference spectrum was produced.[13]

McLean *et al.*[3,5] measured the rate of production of $^{14}CO_2$ from $^{14}CCl_4$ both *in vitro* and *in vivo* and noted that the increase in toxicity of CCl_4 produced by phenobarbitone pretreatment was proportional to the increase in the rate of metabolism of CCl_4 to CO_2. Garner and McLean[5] concluded that "the toxic effects of CCl_4 cannot be related to the action of unaltered CCl_4, and strengthen the view that CCl_4 must be metabolized to cause cell damage". It would seem, therefore, that one way of preventing the toxic manifestations of CCl_4 would be by inhibiting its metabolic conversion to CO_2.

Numerous reports have appeared in the literature concerning substances that are able to ameliorate the toxic effect of CCl_4. Recently, Cawthorne *et al.*[14] found that 6-ethoxy 2,2,4-trimethyl-1-2-dihydroquinoline (ethoxyquin), when given 48 hr before

CCl_4, not only prevented the toxicity of CCl_4 in normal rats, but also reversed the potentiating effect of DDT. The LD_{50} of CCl_4 in the DDT-pretreated rat was 2 ml/kg. Further pretreatment of the DDT-treated rat with ethoxyquin raised the LD_{50} of CCl_4 to 6 ml/kg. The LD_{50} of CCl_4 in nonpretreated rats was 8 ml/kg.

In the present paper, we report the effects of ethoxyquin on the metabolism of CCl_4 to CO_2 *in vitro* and *in vivo*.

MATERIALS AND METHODS

Rats. Male and female Wistar rats (150–200 g) were obtained from Scientific Product Farms, Ash, Kent. Unless stated otherwise, they were fed the stock-pelleted diet FFG (E. Dixon & Sons, Ware, Herts.), and were housed in plastic cages with wire-meshed floors. The 3% casein diet was as described by Cawthorne *et al.*[14]

Dosage of animals. DDT was a commercial sample, 99% pure. It was dissolved in methyl oleate at a concentration of 100 mg/ml and given to rats, as a single subcutaneous injection (100 mg/kg), 7 days before they were treated with CCl_4.

Ethoxyquin (500 mg/kg) and BHT (500 mg/kg) were given orally, as 50% (w/v) solutions in methyl oleate, to rats 48 hr before they were given CCl_4.

Metabolism of carbon tetrachloride in vivo. $^{14}CCl_4$ was purchased from Radiochemical Centre, Amersham, Bucks., and was diluted with carrier CCl_4 (Analar grade; British Drug Houses, Poole, Dorset). Rats were given 2·0 ml/kg of $^{14}CCl_4$ (sp.act. 5 μCi/ml) intragastrically. Two rats were placed in a metabolism cage (Metabowl, Jencons Ltd., Hemel Hempstead, Herts.), and air was drawn through the cage at a flow-rate of 500 ml/min. The expired air was passed through two towers containing toluene to absorb unchanged $^{14}CCl_4$ and then through two towers containing a 10% (v/v) solution of 2-phenylethylamine in methanol to absorb $^{14}CO_2$. Samples were taken from each tower at 2-hr intervals for 5 hr and placed in counting vials containing 10 ml of butyl PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in toluene (6 g/l.): radioactivity was measured in an Intertechnique ABAC SL40 spectrometer. Correction for quenching was performed by the external standard channels–ratio method.

Metabolism of carbon tetrachloride in vitro. A 10 per cent liver homogenate was prepared in 0·25 M sucrose buffered to pH 7·4 with 0·10 M sodium phosphate and 0·001 M EDTA. After filtering through gauze, the homogenate (1 ml) was placed in a Warburg flask together with 3 ml of medium having the composition (final concentrations): KH_2PO_4 buffer pH 7·4, 83·3 mM; $MgCl_2$, 3·3 mM; NADPH, 0·21 mM. The flasks were then placed in an ice-bath and $^{14}CCl_4$ (5 μl, 2·2 $\times$ 10^6 dis/min) placed in the side-arm. The $^{14}CCl_4$ was distilled into the main body of the closed flask by gently warming the side-arm. The flasks were incubated at 37° for 20 min with air as gas phase and the $^{14}CO_2$ was collected in 0·4 ml of 20% (w/v) KOH, which was contained in the centre wells of the flasks. The enzyme reaction was stopped by the addition of 1 ml of 25% (w/v) trichloroacetic acid. Carrier CO_2 was provided by adding solid Na_2CO_3 to the side-arm, followed by 1 ml of trichloroacetic acid. The flasks were then reincubated for 30 min to allow complete absorption of the CO_2 by the KOH solution. The contents of the centre well were transferred to a tube containing $BaCl_2$ and the resulting precipitate of $BaCO_3$ was washed twice with both water and acetone before being suspended in a mixture of the xylene-based scintillator NE 213 (Nuclear Enter-

TABLE 1. EFFECT OF DDT-PRETREATMENT AND ETHOXYQUIN-PRETREATMENT ON THE RATE OF CONVERSION OF $^{14}CCl_4$ TO $^{14}CO_2$ BY RAT LIVER HOMOGENATES

Exp.	Pretreatment	No. animals	Rat wt (g)	Liver wt (% body wt)	CCl_4 metabolizing enzyme: μmole ^{14}CO formed /g liver/hr	CCl_4 metabolizing enzyme: Control (%)
1	None	8	230 ± 14	4·2 ± 0·5	0·73 ± 0·11	(100)
	DDT	8	238 ± 24	4·4 ± 0·5	1·18 ± 0·28*	162
	Ethoxyquin	8	236 ± 21	5·2 ± 0·3†	0·58 ± 0·13	79
2	None	4	214 ± 19	3·7 ± 0·5	1·45 ± 0·36	(100)
	DDT	6	235 ± 35	4·5 ± 0·3*	2·08 ± 0·71	154
	DDT + ethoxyquin	6	231 ± 19	5·5 ± 0·4†‡	1·93 ± 0·15	143
3	Low protein diet	6	153 ± 8	4·4 ± 0·5	0·08 ± 0·06	(100)
	Low protein diet + DDT	6	154 ± 12	5·3 ± 0·7§	0·23 ± 0·08	287
	Low protein diet + DDT + ethoxyquin	6	153 ± 8	6·3 ± 0·9†	0·26 ± 0·08§	325

Results are expressed as mean ± S. D. The low-protein diet was given for 7 days before the enzyme assays. DDT (100 mg/kg) was given subcutaneously and ethoxyquin (500 mg/kg) was given orally 1 week and 48 hr, respectively before the rats were killed. Livers were combined in pairs, homogenates were prepared and incubated in quadruplicate with 5 μl ($2·2 \times 10^6$ dis/min) of $^{14}CCl_4$ as described in the Methods.

* Significantly greater than rats given no pretreatment ($P < 0·01$).

† Significantly greater than rats given no pretreatment ($P < 0·001$).

‡ Significantly greater than rats given DDT alone ($P < 0·001$).

§ Significantly greater than rats given no pretreatment ($P < 0·05$).

prises, Edinburgh, Scotland) and Triton X-100 (C. Lennigg, Jarrow, Northumberland) (2:1, v/v). Correction for quenching was made by the channels–ratio method.

In all experiments, control incubations without tissue were done to assess the radiochemical contamination of $^{14}CCl_4$ with $^{14}CO_2$, nonenzymic conversion of $^{14}CCl_4$ to $^{14}CO_2$ and contamination of the $Ba^{14}CO_3$ precipitate with $^{14}CCl_4$.

Binding spectrum of carbon tetrachloride. The spectral changes produced by the addition of CCl_4 to a suspension of microsomes were recorded in a Unicam SP 8000 spectrophotometer. Each cell contained microsomal suspension in 0·10 M KCl buffered with 0·05 M sodium phosphate, pH 7·4.

Hepatic neutral lipids. The neutral lipid content of liver was determined by the method described by Bunyan *et al.*[15]

Protein. Protein was measured by the method of Lowry *et al.*[16] using bovine serum albumin as standard.

EXPERIMENTAL AND RESULTS

Effect of ethoxyquin on the metabolism of carbon tetrachloride by rat liver homogenates. The pretreatment of male rats with DDT (100 mg/kg, given subcutaneously 7 days prior to the enzyme assay) produced a marked increase in the activity of the hepatic enzyme system that converts CCl_4 to CO_2. The enzyme activity was low in rats that had been previously fed a diet low in protein for 7 days but, if these rats were also given DDT, the enzyme activity was increased. All these findings are in agreement with the results of Seawright and McLean.[3]

Some rats were given a single oral dose of ethoxyquin (500 mg/kg) 48 hr before they were killed for the enzyme assay. The ethoxyquin treatment had no effect on the rate of conversion of CCl_4 to CO_2 in normal rats, in DDT-pretreated rats or in rats fed the low-protein diet and given DDT (Table 1).

Effect of ethoxyquin on the metabolism of carbon tetrachloride by the rat in vivo. In experiment 1 (Table 2), the effect of ethoxyquin on the metabolism and hepatotoxicity of CCl_4 was examined. Ethoxyquin produced a small increase in the mean rate of elimination in the expired air of both $^{14}CCl_4$ and $^{14}CO_2$. On the other hand, ethoxy-

TABLE 2. EFFECT OF ETHOXYQUIN-PRETREATMENT ON THE EXPIRATION OF $^{14}CCl_4$ AND $^{14}CO_2$ IN THE RAT

Exp.	Treatment	No. in group	Dose expired (%)		Liver neutral lipid (mg/g)
			$^{14}CCl_4$	$^{14}CO_2$	
1	None	4	28·1 ± 1·3	0·20 ± 0·05	28·2 ± 9·0
	Ethoxyquin	4	39·4 ± 5·1	0·34 ± 0·01	23·3 ± 5·7
2	Low-protein diet + DDT	6	30·1 ± 9·4	0·29 ± 0·08	39·4 ± 7·6
	Low-protein diet + DDT + ethoxyquin	6	43·4 ± 6·1	0·48 ± 0·05*	25·5 ± 4·9†

The low-protein diet was fed for 7 days before the male rats were given CCl_4. DDT (100 mg/kg) was given as a single subcutaneous injection 7 days before CCl_4 dosage and ethoxyquin (500 mg/kg) was given orally 48 hr before the CCl_4. The rats were given CCl_4 (2 ml/kg); 5 μCi/ml in a mixture with liquid paraffin (2 ml/kg) and immediately placed in pairs in the metabolism cage. After 6 hr, the rats were removed from the cage, killed, their livers and samples taken for the determination of neutral lipid content. Results are given as mean ± S. D.

* Significantly greater than control rats not given ethoxyquin ($P < 0·05$).

† Significantly less than control rats not given ethoxyquin ($P < 0·01$).

quin inhibited the CCl_4-induced accumulation of neutral lipid. None of the effects were significant in this experiment.

In a second experiment, rats were fed the 3% casein diet and given an oral dose of DDT. Treatment of these rats with ethoxyquin produced a significant increase in the rate of expiration of $^{14}CO_2$ ($P < 0{\cdot}05$) and a significant reduction in the accumulation of liver lipid ($P < 0{\cdot}01$).

Effect of various pretreatments of the rat on the binding spectrum of carbon tetrachloride with liver microsomes. When CCl_4 was added to a suspension of liver microsomes from control rats, a typical type I binding spectrum with a peak at 388 nm and a trough at 422 nm was produced. In our experiments, the mean ΔA value between 388 and 422 nm was 0·026/mg microsomal protein. Some rats were given a phenobarbitone solution (1 mg/ml) to drink for 7 days before they were killed. When CCl_4 was added to a microsomal suspension from phenobarbitone-treated rats a much larger ΔA value was obtained (0·067/mg protein) and the peak was now at 385 nm and the trough at 423 nm. However, when rats that had been treated 48 hr previously with either butylated hydroxytoluene (500 mg/kg) or ethoxyquin (500 mg/ kg) were used as the source of hepatic microsomes the ΔA values/mg microsomal protein were 0·023 and 0·022, respectively.

DISCUSSION

The discovery by McLean *et al.*[3,5] of a correlation between the toxicity of CCl_4 and the activity of the hepatic microsomal enzymes that metabolize CCl_4 to CO_2 suggests that a metabolite of CCl_4 is the true hepatotoxic agent. If this idea is correct, it follows that CCl_4 hepatotoxicity might be prevented by compounds that inhibit CCl_4 metabolism. Indeed, it has been shown that SKF 525-A prevents CCl_4-induced necrosis[14] and disulfiram (tetraethyl thiuram disulphide) prevents $CHCl_3$ toxicity as well as inhibiting drug-metabolizing enzyme activity.[19,20] However, in order to prevent the toxicity of the halohydrocarbons, both SKF 525-A and disulfiram have to be given within a few hours of the CCl_4 dose.

In a recent study,[14] it was found that both ethoxyquin and BHT had a remarkable effect on CCl_4 hepatotoxicity. Given 48 or 72 hr before CCl_4, both substances prevented the hepatotoxicity; given 6 hr before CCl_4, ethoxyquin was without effect and BHT exacerbated the CCl_4-induced hepatotoxicity. Furthermore, 48 hr after dosage to rats, BHT and ethoxyquin can barely be detected in the liver. These findings lead us[14] to the conclusion that ethoxyquin and BHT are unlikely to prevent CCl_4-induced hepatotoxicity by acting directly as inhibitors of CCl_4 metabolism. Nevertheless, the possibility exists that ethoxyquin and BHT might modify the activity of the enzymes that metabolize CCl_4 to CO_2 in a more indirect manner.

The experiments described here, however, demonstrate that ethoxyquin treatment does not result in a reduced conversion of CCl_4 to CO_2 *in vitro* or *in vivo*. Furthermore, neither ethoxyquin-pretreatment nor BHT-pretreatment affected the binding spectrum produced by CCl_4 with liver microsomes.

In our earlier publication,[14] we found that ethoxyquin was an inducer of drug metabolizing enzymes and we suggested that ethoxyquin might selectively increase the activity of a CCl_4-detoxification pathway. We consider that ethoxyquin might accomplish this action in one of two ways. Firstly, it might induce the activity of a metabolic

pathway parallel to that induced by pretreatment with phenobarbitone or DDT. Although both pathways would appear to terminate in CO_2, the ethoxyquin-induced pathway would not appear to involve the toxic intermediate. Secondly, ethoxyquin might accelerate the further metabolism of the hepatotoxic intermediate and thus could prevent the occurrence of a lethal concentration of the hepatotoxic intermediate. It has recently been shown[21-23] that pretreatment of rats with 3-methylcholanthrene (MC) partially blocks the hepatotoxicity of CCl_4 and bromobenzene, and Reid *et al.*[21] conclude it is probable that 3-MC administration increases the overall rate of [^{14}C]-bromobenzene metabolism in rats but reduces the metabolic pathway leading to formation of a toxic metabolite capable of binding covalently to tissue macromolecules. It seems possible that ethoxyquin and 3-methylcholanthrene might affect CCl_4 toxicity by similar mechanisms.

Acknowledgements—We are indebted to Mr. T. R. Clarke for providing excellent technical assistance. We wish to thank Monsanto Chemical Co., Ruabon, Denbighshire, Wales, for a gift of ethoxyquin. Part of this work has been presented in a Ph.D. thesis to the University of London by M. A. Cawthorne.

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