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PROTECTION BY DIETHYLDITHIOCARBAMATE AGAINST CARBON TETRACHLORIDE LETHALITY IN RATS AND AGAINST CARBON TETRACHLORIDE-INDUCED LIPID PEROXIDATION *IN VITRO**

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Abstract—Diethyldithiocarbamate provides excellent protection against carbon tetrachloride-induced lethality when administered 1 hr prior to the poison. Lipoperoxidation of liver microsome preparations and the pro-oxidant effect of carbon tetrachloride *in vitro* are inhibited by low concentrations of diethyldithiocarbamate. On the other hand, NADPH-cytochrome *c* reductase activity is not affected by addition of diethyldithiocarbamate. These results suggest that the action of the protective agent is not at the level of the flavoprotein of the microsomal electron transport system but elsewhere in the process of lipid peroxidation.

THE PROTECTIVE effect of diethyldithiocarbamate (DEDTC) against lethal effects of radiation has been reported.[1,2] Many hypotheses of the actions of radioprotectors have been postulated.[3] One hypothesis proposes that radioprotectors act as radical "scavengers", that they compete with free radicals produced by the radiolysis of water for radiosensitive sites of biologically important molecules.[3] Recently DEDTC has been shown to ameliorate the effects of CCl_4 on the liver of mice.[4] The juxtaposition of these two ideas is interesting in light of a current hypothesis of CCl_4 liver injury.[5,6] According to this hypothesis, the toxicity of CCl_4 is a consequence of its metabolism to trichloromethyl free radical and monatomic chlorine free radical. These highly reactive species have been postulated to attack structural lipids of the endoplasmic reticulum resulting in peroxidation of lipids, a process which also involves free radicals.[7] Perhaps, therefore, knowledge of the action of DEDTC and other radioprotective agents can be applied to the situation of CCl_4-induced liver injury.

Previously reported studies on protection of DEDTC against CCl_4 toxicity involved measurement of serum glutamic-oxaloacetic transaminase levels, hexobarbital sleeping time and bromsulfophthalein retention.[4] Other works have compared hepatoprotective effects of dithiocarbamate derivatives and related compounds, as evidenced by reduction in plasma transaminase levels.[8] None to our knowledge have demonstrated a protection against death. This paper reports our experiments on the protection of

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DEDTC against the lethality of CCl_4. Since the toxicity of CCl_4 may be related to induction of peroxidative decomposition of liver microsomal lipids, the effect of DEDTC on lipid peroxidation *in vitro* was also studied.

METHODS

Animals and lethality studies. Male rats of the Sprague–Dawley strain, weighing 150–220 g, were used. The animals were fasted overnight before administration of drugs and were re-fed approximately 6 hr after administration of carbon tetrachloride. Water was given *ad lib.* throughout the experiment. The animals were observed for 7 days to determine lethality of the CCl_4.

TABLE 1. PROTECTION BY VARIOUS DOSES OF DIETHYLDITHIOCARBAMATE (DEDTC) AGAINST LETHALITY OF CCl_4

Dose of DEDTC (mg/kg body wt)	No. of animals	No. of survivors	Survival (%)
	Initial survey		
0	15	0	0
10	9	1	11
25	9	4	44
50	13	6	46
100	10	9	90
300	5	5	100
	Confirmatory experiment		
0	10	0	0
100	10	10	100

* Conditions: DEDTC in saline or saline alone was injected i.p. 1 hr before administration of CCl_4. CCl_4 was administered to all of the rats intragastrically in mineral oil under light ether anaesthesia at a dose of 5·0 ml of CCl_4/kg of rat body wt.

Diethyldithiocarbamate, as sodium salt, was obtained from Sigma Chemical Co. It was dissolved in 0·9% NaCl to appropriate concentrations and administered intraperitoneally at various dosages (see Table 1). In all cases, the volume of saline administered was 5·0 ml/kg body wt. Control animals received an injection of 0·9% saline. Carbon tetrachloride was dissolved in mineral oil (CCl_4–mineral oil, 2:1) and administered by stomach tube under light ether anaesthesia. The dose of CCl_4 was 5·0 ml/kg body wt.

Studies in vitro. Rat liver microsome-supernatant or microsome fractions were obtained and incubated as previously reported.[9] The degree of lipid peroxidation was determined by measuring the yield of malonic dialdehyde (MDA) with thiobarbituric acid.[10] Acceleration of lipid peroxidation by carbon tetrachloride, i.e. the pro-oxidant effect, was produced in microsome-supernatant fractions supplemented with NADPH (final concentration 0·2 mM) and with reduced glutathione (GSH) (final concentration 0·3 mM).[9] Microsomal NADPH-cytochrome *c* reductase activity was measured by observing the increase in absorption at 550 nm produced by reduction of cytochrome *c*.[11]

RESULTS

DEDTC protection against CCl_4 lethality. An initial exploratory experiment was conducted to determine the range of doses of DEDTC effective against CCl_4 lethality. DEDTC alone, at a dose of 1·0 g/kg body weight, killed three of five rats. This high dose was not further studied. At does of 300 and 100 mg of DEDTC/kg body wt, excellent protection against CCl_4 lethality was observed (Table 1). Lower doses were less effective. On the basis of this initial survey, a second experiment was carried out with ten animals per group. The control group received a saline injection 1 hr before CCl_4; the protected group received 100 mg of DEDTC/kg body wt 1 hr before CCl_4. DEDTC was 100 per cent effective in protecting rats from the lethality of CCl_4 (see confirmatory experiment, Table 1).

TABLE 2. DIETHYLDITHIOCARBAMATE (DEDTC) INHIBITION *in vitro* OF LIPID PEROXIDATION IN THE LIVER MICROSOME-SUPERNATANT FRACTION*

Additions to liver microsome-supernatant fraction		Malonic dialdehyde production (μg/eq. g liver)
Exp. 1	Control (no DEDTC)	46·7
	30 mM DEDTC	0
	10 mM DEDTC	0·7
	3 mM DEDTC	1·0
	1 mM DEDTC	0·5
	0·3 mM DEDTC	0·6
Exp. 2	Control	58·4
	100 μM DEDTC	0
	30 μM DEDTC	1·3
	10 μM DEDTC	0·1
	3 μM DEDTC	0
Exp. 3	Control	51·8
	1·0 μM DEDTC	0·4
	0·3 μM DEDTC	57·9
	0·1 μM DEDTC	58·1
	0·03 μM DEDTC	55·9
	0·01 μM DEDTC	55·9

* Lipid peroxidation was measured as malonic dialdehyde production. Incubation was carried out for 60 min at 38° at a concentration of the liver microsome-supernatant fraction equal to the equivalent of 25 mg wet wt of liver/ml of medium (0·155 M NaCl buffered with 0·05 M phosphate, pH 6·5). DEDTC concentrations are the final concentrations, millimolar or micromolar, as indicated.

Experiments in vitro. Incubation of the liver microsome-supernatant fraction results in peroxidation of constituent lipids as evidenced by production of MDA.[9] Lipids of this liver microsome-supernatant system peroxidize without addition of NADPH. Addition of DEDTC to the incubation mixture inhibits the peroxidation. This inhibition is observed with concentrations of DEDTC ranging from 30 mM to as low as 1·0 μM. At concentrations of 0·3 μM or lower, DEDTC does not inhibit the production of MDA (Table 2).

TABLE 3. DIETHYLDITHIOCARBAMATE (DEDTC) INHIBITION *in vitro* OF NADPH-LINKED LIPID PEROXIDATION IN THE LIVER MICROSOME FRACTION*

Additions to liver microsome fraction	Malonic dialdehyde production (μg/eq. g liver)
Control (microsomes only)	2·0
0·1 mM NADPH	58·1
0·1 mM NADPH + 3·0 μM DEDTC	0·3
0·1 mM NADPH + 1·0 μM DEDTC	1·4
0·1 mM NADPH + 0·3 μM DEDTC	55·8
0·1 mM NADPH + 0·1 μM DEDTC	58·8

* Lipid peroxidation was measured as malonic dialdehyde production. Incubation was carried out for 20 min at 38° at a concentration of the liver microsome fraction equal to the equivalent of 25 mg wet wt of liver/ml of medium (0·155 M NaCl buffered with 0·05 M phosphate, pH 6·5).

Unsupplemented microsomes when incubated at 38° produce little MDA.[9] When NADPH is added there is a pronounced production of MDA within 20 min. Again, addition of DEDTC at concentrations of 1·0 μM or greater inhibits the lipid peroxidation (Table 3); 0·3 μM DEDTC is too low to be effective.

MDA production in a hepatic microsome-supernatant fraction supplemented with 0·2 mM NADPH and 0·3 mM GSH is very low (Fig. 1). Addition of 1 μl of CCl_4/ml greatly augments MDA production in this system. This pro-oxidant effect of CCl_4 is totally inhibited by addition of DEDTC at a concentration of 3 μM. In contrast, DEDTC has no inhibitory action against microsomal NADPH-cytochrome *c* reductase (Table 4).

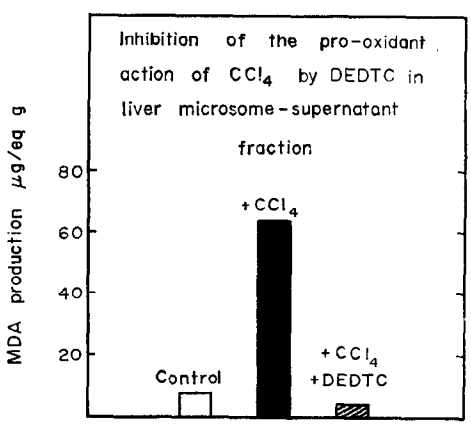

FIG. 1. Inhibition of the NADPH-linked pro-oxidant action of CCl_4 by diethyldithiocarbamate (DEDTC) in liver microsome-supernatant fraction. Lipid peroxidation was measured as malonic dialdehyde (MDA) production. Incubation was carried out for 45 min at 38° at a concentration of the microsome-supernatant fraction equal to the equivalent of 25 mg wet wt of liver/ml of medium (0·155 M NaCl buffered with 0·05 M phosphate, pH 6·5). All of the microsome-supernatant fractions were incubated with 0·2 mM NADPH and 0·3 mM GSH. CCl_4 was added directly to the medium at 1 μl/ml. The concentration of DEDTC was 3·0 μM.

TABLE 4. EFFECT OF DIETHYLDITHIOCARBAMATE (DEDTC) *in vitro* ON LIVER MICROSOMAL NADPH-CYTOCHROME *c* REDUCTASE ACTIVITY

Addition to liver microsome fraction	NADPH-cytochrome reductase activity* (ΔA_{550}/min/mg protein)
All additions, except DEDTC (control)	0·290 ± 0·010
All additions, plus 3·0 μM DEDTC	0·286 ± 0·011

* Measured as the increase in absorbance at 550 nm produced by reduction of cytochrome *c* by NADPH (for details of assay, see Ref. 11).

DISCUSSION

The significance of these experiments may be considered from a number of viewpoints. First, in a general context, the protective properties of DEDTC bring together two large bodies of knowledge, that concerning radiation injury on one hand and that concerning CCl_4 hepatotoxicity on the other. The fact that DEDTC has a protective action in both situations suggests a commonality of mechanism at the molecular level between radiation injury and haloalkane toxicity. The common mechanism may involve production of free radicals, but this hypothesis has not been proven.

More specifically, these experiments are significant because the remarkable protection by DEDTC opens up new opportunities for studying the mechanism of CCl_4 toxicity. For example, the question arises as to whether DEDTC protects against the lipid peroxidation known to occur with great rapidity in liver endoplasmic reticulum of rats given CCl_4.[12] Also, the effect of DEDTC on incorporation of ^{14}C from $^{14}CCl_4$ into microsomal lipids when $^{14}CCl_4$ is administered,[13] and possible effects of DEDTC on retardation of conversion of CCl_4 to CO_2 should be investigated. Studies concerning these questions are now underway in this laboratory.

Investigation of the action of DEDTC may provide insights into the molecular mechanisms of free radical tissue injury. By use of *in vitro* systems, it has been established that peroxidation of microsomal lipid is linked to the NADPH oxidase–drug-metabolizing system resident in liver microsomes.[14–17] Additional work has indicated that electron flow past the flavoprotein stage of this system is necessary for both lipid peroxidation and the pro-oxidant action of CCl_4 to occur.[9,18] The latter conclusion was based on the finding that, when electron flow is short-circuited by addition of ferricyanide or cytochrome *c*, lipid peroxidation and the pro-oxidant effect of CCl_4 are inhibited, even though the flavoprotein is oscillating between its oxidized and reduced form. Inhibitors of lipid peroxidation may act at the flavoprotein site, at sites elsewhere in the mixed function chain of enzymes, or directly on the autocatalytic phase of the lipid peroxidation itself, e.g. as free radical scavengers.

Our results (Fig. 1, and Tables 2 and 3) indicate complete inhibition of microsomal lipid peroxidation *in vitro* by DEDTC in low concentrations. The flavoprotein, measured as NADPH-cytochrome *c* reductase, is not inhibited by DEDTC (Table 4). These results permit the conclusion that inhibition of lipid peroxidation by DEDTC is not being exerted at the flavoprotein site, but elsewhere in the overall process of microsomal lipid peroxidation.

REFERENCES

1. Z. M. BACQ, A. HERVE and P. FISCHER, *Bull. Acad. r. Med. Belg.* **18,** 226 (1953).
2. D. W. VAN BEKKUM, *Acta Physiol. Pharmac. néerl.* **4,** 508 (1956).
3. Z. M. BACQ, *Chemical Protection Against Ionizing Radiation*, pp. 216–226. Charles C. Thomas, Springfield, Illinois (1965).
4. P. LANGE and F. JUNG, *Acta biol. med. Germ.* **27,** 425 (1971).
5. R. O. RECKNAGEL, *Pharmac. Rev.* **19,** 145 (1967).
6. T. F, SLATER, *Free Radical Mechanisms in Tissue Injury*, pp. 91–170. Pion, London (1972).
7. R. O. RECKNAGEL and A. K. GHOSHAL, *Lab. Invest.* **15,** 132 (1966).
8. T. SAKAGUCHI, H. NISHIMURA, K. MASUDA, I. TSUGE, K. ONISHI and H. TATSUMI, *Biochem. Pharmac.* **15,** 756 (1966).
9. E. A. GLENDE, JR. and R. O. RECKNAGEL, *Exp. molec. Path.* **11,** 172 (1969).
10. A. K. GHOSHAL and R. O. RECKNAGEL, *Life Sci.* **4,** 1521 (1965).
11. E. A. GLENDE, JR., *Biochem. Pharmac.* **21,** 2131 (1972).
12. K. S. RAO and R. O. RECKNAGEL, *Exp. molec. Path.* **9,** 271 (1968).
13. K. S. RAO and R. O. RECKNAGEL, *Exp. molec. Path.* **10,** 219, (1969).
14. H. E. MAY and P. B. MCCAY, *J. biol. Chem.* **243,** 2288 (1968).
15. H. E. MAY and P. B. MCCAY, *J. biol. Chem.* **243,** 2296 (1968).
16. B. K. TAM and P. B. MCCAY, *J. biol. Chem.* **245,** 2295 (1970).
17. P. HOCHSTEIN and L. ERNSTER, *Biochem. biophys. Res. Commun.* **12,** 388 (1963).
18. E. A. GLENDE, JR. and R. O. RECKNAGEL, *Fedn Proc.* **29,** 755 (1970).