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REPAIR OF RAT LIVER DNA *IN VIVO* DAMAGED BY ETHYLENE DIBROMIDE

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Abstract—Tube feeding of [^{14}C]ethylene dibromide (EDB) to non-fasted rats resulted in the incorporation of the radioactivity into liver DNA, RNA and protein. Using alkaline and neutral sucrose gradients, it was observed that administration of the pesticide to non-fasted rat caused slower sedimentation of liver DNA in alkaline and not in neutral sucrose gradients. Slower sedimentation of liver DNA in alkaline sucrose gradients was apparent within 2 hr after the administration of a dose of 22 mg/100 g or 4 hr after a dose of 7.5 mg/100 g of body weight. Using a dose of 7.5 mg/100 g, the EDB-induced liver DNA damage was repaired significantly by 17.5 hr and almost completely by 96 hr. Administration of diethyldithiocarbamate, a free radical scavenger, did not inhibit liver DNA damage caused by EDB.

Ethylene dibromide (EDB) is a pesticide used either separately or mixed with other haloalkane fumigants like ethylene dichloride and carbon tetrachloride. This agent has been found to be toxic to animals [1, 2], to cause a decrease in the laying of eggs in chicks [3] and to be spermicidal in bulls [4]. Recently it has also been reported to be carcinogenic [5] and mutagenic [6].

It has been demonstrated that carcinogens interact with DNA of several organs and induce slow sedimentation of DNA in alkaline and/or neutral sucrose gradients indicative of single and/or double strand breaks respectively [7–16]. Therefore it was of interest to determine whether EDB would interact with liver DNA *in vivo* and induce DNA damage that could be measured by its slow sedimentation in alkaline and/or neutral sucrose gradients.

The results presented in this paper show that (1) EDB interacts with liver DNA, RNA and protein and induces single strand breaks in liver DNA; (2) the strand breaks are largely repaired by 96 hr after the administration of the halo-alkane; and (3) pretreatment of the animal with diethyldithiocarbamate (DEDTC), a free radical scavenger, does not prevent the induction of liver DNA strand breaks caused by EDB.

MATERIALS AND METHODS

White male rats of Wistar strain (Carworth Farms) weighing about 130–160 g were used in all experiments. Thymidine-[methyl-^{3}H] (sp. act. 20 Ci/m-mole) and [^{14}C(U)]EDB (sp. act. 20 mCi/m-mole) were purchased from New England Nuclear, Boston, MA. Unlabeled EDB was obtained from Aldrich Chemicals, Milwaukee, WI; and DEDTC and pronase were obtained from Sigma Chemical Co., St. Louis, MO. Cellulose nitrate tubes (Beckman Instruments,Inc., Fullerton, CA) and dialysis tubing (A. H. Thomas, Philadelphia, PA) were treated with 0.5% boiling solution of EDTA (pH 7.5) for 10 min and rinsed with distilled water before use. Liver DNA was labeled by injecting thymidine-[methyl-^{3}H] during the peak of DNA synthesis after partial hepatectomy [8]. The animals were used after a minimum recovery period of 2 weeks, by which time the liver had returned to a quiescent state [17].

After appropriate treatments, rats were killed by decapitation, livers excised and the size of the liver DNA was measured by sedimentation in alkaline sucrose density gradient. The preparation of alkaline and neutral sucrose gradients, suspension of liver nuclei, lysis of nuclei and release of DNA on the top of sucrose gradient and other technical details were carried out as described previously [8]. The alkaline lysing agent consisted of 0.3 M NaCl, 0.03 M EDTA, 0.5% sodium dodecyl sulfate and 0.1 M Tris, the pH of which was adjusted to 12.5 with 5 N NaOH [18]. Neutral lysing agent was similar to the alkaline lysing agent except that the pH was 7.5 and contained autodigested pronase (2 mg/ml of lysing solution [8]).

Alkaline sucrose gradients were calibrated using DNA from T_4, T_7 and SV40 nicked circles. Molecular weight values higher than T_4 phage-DNA are extrapolated from the sedimentation patterns of DNA of SV40, T_7 and T_4 phages [19].

Incorporation of radioactivity into liver DNA, RNA and protein after the administration of [^{14}*C(U)*]*EDB.* [^{14}C(U)]EDB was mixed with unlabeled EDB to give a specific activity of 15.6 μCi/mg. Labeled EDB was dissolved in corn oil and was given by stomach tube (12 mg/100 g of body weight). Four hours later the rats were killed by decapitation, livers excised, weighed and rinsed in 0.25 M sucrose containing 0.05 M Tris, pH 7.6, 0.025 M KCl and 0.005 M $MgCl_2 \cdot 6H_2O$ (TKM) and homogenized in 2 vol. of 0.25 M sucrose

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containing TKM, using a Potter–Elvehjem homogenizer with a clearance of 0.006 to 0.009 in. between Teflon pestle and glass chamber. The liver was homogenized with ten strokes (one up and one down movement per stroke). The homogenate was centrifuged at 3000 rev/min in an International Centrifuge model PR-6 at 4° for 10 min to pellet the nuclei. After pelleting the mitochondria at 12,000 rev/min in a Sorvall Centrifuge model RC-2, the post-mitochondrial supernatant was centrifuged at 100,000 *g* for 4 hr in a Spinco ultracentrifuge at 4° to obtain microsomal pellet and the supernatant cytoplasmic fraction. The nuclear and microsomal pellets were rinsed with 0.25 M sucrose containing TKM and repelleted. The nuclear DNA was isolated and purified either by the procedure described by Kirby and Cook [20] or using CsCl [21].

Ribosomal and cytoplasmic RNA and nuclear, microsomal and cytoplasmic proteins were prepared as described by Schneider [22]. The radioactivity in these fractions was determined as detailed earlier [8]. DNA was determined by the diphenylamine method [23], RNA by the orcinol method [24] or by measuring absorption at 260 nm and protein by the method described by Lowry *et al.* [25] or by the fluorescence method [26].

RESULTS

The results on the incorporation of radioactivity into liver DNA, RNA and protein after the administration of [^{14}C(U)]EDB are presented in Table 1. The incorporation of radioactivity was highest in the protein fraction followed by RNA and DNA. The radioactivity in DNA was hydrolyzable to an extent of 80–90 per cent by pancreatic DNase. Acid hydrolysates (0.1 N HCl at 90° for 60 min) of ^{14}C-labeled rat liver DNA gave three major and two minor u.v. quenching radioactive spots on thin-layer chromatograms (Mn 200 cellulose; thickness 250 μm; Analtech, Inc., Newark, DE) developed in isopropanol–conc. HCl–water (170:41:39) solvent system for 4 hr. The chemical nature of these compounds is not known and is being studied. Even though the nature of the interaction of EDB with liver DNA is not known at present, on a molar basis its interaction with DNA (5.68×10^6 mole nucleotides/mole EDB) approaches that obtained with several alkylating agents [27]. The next series of experiments was designed to determine whether EDB, like other alkylating agents, causes slow sedimentation of liver DNA in alkaline and/or neutral sucrose gradients.

Table 1. Incorporation of radioactivity into liver DNA, RNA and protein after the administration of [^{14}C(U)]EDB*

Cellular fraction	Incorporation of radioactivity (dis./min/mg)
Nuclear DNA	3,000; 2,700†
Ribosomal RNA	9,000
Cytoplasmic RNA	9,800; 8,400
Nuclear protein	14,000
Microsomal protein	10,000
Cytoplasmic protein	20,000; 30,000

* Livers of two rats were pooled and each value represents one determination. Other details are described in Materials and Methods.

† DNA samples were found to contain 2–3% protein and hence the values were corrected for the nuclear protein contamination.

As can be seen from Table 2, while most of the liver DNA of control rats sedimented in the molecular weight range of $> 3.2 \times 10^9$ daltons, that from rats treated with EDB (7.5 mg/100 g) for 4 hr had a lower molecular weight and was heterodisperse in alkaline sucrose gradients. The acid-precipitable radioactivity in the gradients fractions was essentially free of protein and RNA [8] and was characterized as DNA by its stability to alkali (0.3 N NaOH), insensitivity to pronase and susceptibility to DNase digestion. It was further judged to be single stranded by physical and electron microscopic methods as well as by its susceptibility toward S_1 nuclease [19].

The data presented in Table 2 also indicate that the slow sedimentation of liver DNA seems to be little affected by increasing the dose of EDB above 7.5 mg/100 g of body weight. This lack of progression of DNA damage with increasing the dose of EDB

Table 2. Sedimentation patterns of hepatic DNA in alkaline sucrose gradients after the administration of different doses of EDB

EDB administered* (mg/100 g body wt)	No. of rats	Distribution of liver DNA in alkaline sucrose gradients (% of total radioactivity)		
		Fractions and corresponding mol. wt		
		1–4 ($> 3.2 \times 10^9$)	5–11 (3.2×10^9 to 2.2×10^8)	12–18 ($< 2.2 \times 10^8$)
Control	9	84 ± 3†	11 ± 2	5 ± 2
5	1	73	19	8
7.5	7	39 ± 6	49 ± 5	10 ± 4
10	4	39 ± 6	51 ± 6	10 ± 2
15	7	35 ± 9	49 ± 6	15 ± 5
22	5	35 ± 12	52 ± 7	12 ± 6

* EDB was tube fed at different concentrations, and control rats received equivalent volumes of corn oil. All the rats were killed 4 hours later. Other details are described in Materials and Methods. The numbers in parentheses represent molecular weight ranges.

† Mean ± S.E.

Table 3. Sedimentation patterns of hepatic DNA in alkaline sucrose gradients at different time periods after the administration of EDB

Time after administration of EDB*	No. of rats	Distribution of liver DNA in alkaline sucrose gradients (% of total radioactivity)		
		Fractions and corresponding mol. wt		
		1–4 (>3.2 × 10^9)	5–11 (3.2 × 10^9 to 2.2 × 10^8)	12–18 (<2.2 × 10^8)
Control	9	84 ± 3†	11 ± 2	5 ± 2
30 min	2	90 ± 1	8 ± 1	2 ± 1
1 hr	3	71 ± 13	25 ± 17	5 ± 3
2 hr	5	46 ± 7	46 ± 7	7 ± 3
4 hr	5	35 ± 12	52 ± 7	12 ± 6
24 hr	2	23 ± 2	46 ± 1	31 ± 2

* EDB was tube fed at a dose of 22 mg/100 g of body weight. Details are described in Materials and Methods. The numbers in parentheses represent molecular weight ranges.
† Mean ± S.E.

above 7.5 mg/100 g may be due to the inability of the liver to metabolize higher concentrations of EDB during this time period [2].

In the following experiments, the effect of EDB treatment as a function of time on the sedimentation of liver DNA in alkaline sucrose gradients was determined. To study this aspect, a large dose of EDB (22 mg/100 g of body weight) was chosen. The results presented in Table 3 clearly indicate that the slower sedimentation of liver DNA, an effect that is significant and reproducible, can be observed within 2 hr after the EDB administration, a time at which EDB exerted its maximum effect on lowering hepatic soluble sulfhydryl groups [1].

Liver DNA damage (as measured by sedimentation analysis in alkaline sucrose gradients) with a dose of 22 mg/100 g was progressive with time and persists up to at least 24 hr. Since this dose of EDB induces liver cell necrosis at later time periods in fasted animals [28], repair of EDB-induced DNA damage was studied using lower doses. The results presented in Table 4 indicate that liver DNA damage induced by a non-necrogenic dose of 7.5 mg EDB/100 g was largely repaired by 96 hr after the administration of EDB.

EDB, like CCl_4, causes lipid peroxidation [2, 29]. DEDTC, a free radical scavenger, has been shown to inhibit the lipid peroxidation due to CCl_4 [30]. In addition, recently it has been demonstrated that administration of DEDTC also inhibits the induction of liver DNA strand breaks by dimethylnitrosamine (DMN) by inhibiting the microsomal metabolism of DMN [31, 32]. It was therefore of interest to determine whether DEDTC prevents the EDB-induced liver DNA strand breaks. The results presented in Table 5 indicate that DEDTC did not prevent the liver DNA damage caused by EDB; however, in a very few experiments DEDTC offered partial protection.

DISCUSSION

It is clear from this study that EDB interacts with DNA in addition to RNA and protein. Further, EDB administration causes damage to liver DNA, a lesion that can be monitored by sedimentation of liver DNA in alkaline sucrose gradients. This lesion is largely repaired within 96 hr.

Not much is known about the metabolism of EDB in rat liver. Our observation that DEDTC did not inhibit the EDB-induced strand breaks in liver DNA does not necessarily preclude the involvement of the microsomal cytochrome P-450 system in the metabolism of this pesticide. Although DEDTC inhibited the

Table 4. Repair of hepatic DNA damaged by EDB as analyzed by sedimentation in alkaline sucrose gradients

Time after administration of EDB* (hr)	No. of rats	Distribution of liver DNA in alkaline sucrose gradients (% of total radioactivity)		
		Fractions and corresponding mol. wt		
		1–4 (>3.2 × 10^9)	5–11 (3.2 × 10^9 to 2.2 × 10^8)	12–18 (<2.2 × 10^8)
Control	9	84 ± 3†	11 ± 2	5 ± 2
4	7	39 ± 6	50 ± 4	10 ± 4
17.5	2	65 ± 24	34 ± 23	1
24	3	65 ± 8	30 ± 6	11 ± 5
48	5	64 ± 4	30 ± 3	6 ± 4
96	3	83 ± 5	5 ± 1	11 ± 5

* EDB was given at a dose of 7.5 mg/100 g of body weight. Other details are presented in Materials and Methods. The values in parentheses represent molecular weight ranges.
† Mean ± S. E.

Table 5. Influence of DEDTC on EDB-induced DNA damage as analyzed by sedimentation in alkaline sucrose gradients

Treatment*	No. of rats	Distribution of liver DNA in alkaline sucrose gradients fractions (% of total radioactivity)		
		Fractions and corresponding mol. wt		
		1–4 (>3.2 × 10^9)	5–11 (3.2 × 10^9 to 2.2 × 10^8)	12–18 (<2.2 × 10^8)
Control	9	84 ± 3†	11 ± 2	5 ± 2
EDB	4	40 ± 5	54 ± 4	4 ± 2
DEDTC + EDB	12	42 ± 3	50 ± 3	6 ± 1
DEDTC	3	73 ± 7	18 ± 4	8 ± 4

* DEDTC (300 mg/kg) was given i.p. 45 min prior to the administration of EDB (10 mg/100 g). Corresponding control rats received appropriate volume of vehicle alone. All the rats were killed 4 hr after the administration of EDB. Other details are described in Materials and Methods. The numbers in parentheses represent the molecular weight ranges.
† Mean ± S. E.

metabolism of carbon tetrachloride [30, 33] and dimethylnitrosamine [32] (agents that are presumably metabolized by the microsomal cytochrome P-450 systems [30, 34]), it had hardly any effect on the *N*-demethylation of ethylmorphine, cytochrome content or NADPH–cytochrome P-450 reductase [33, 35]. The possibility of the existence of several distinct cytochrome systems, each specific for the metabolism of a particular group of compounds, must therefore be recognized.

While the nature of the chemical lesion in liver DNA caused by EDB is not known, the observed slow sedimentation of the DNA in alkaline sucrose gradients may be due to strand breaks. EDB can cause strand breaks in liver DNA either by alkylation or by free radical production, and the former mechanism seems more probable. Like many other alkylating agents, EDB alkylates glutathione [36]. It is therefore likely that the radioactivity associated with DNA (Table 1) after [^{14}C]EDB administration represents alkylation of DNA base and/or the phosphodiester backbone either of which can cause DNA strand breaks [27, 37, 38]. Furthermore, EDB was found to be less effective than CCl_4 in inducing lipid peroxidation [2], an agent which produces free radicals [29], and DEDTC, a free radical scavenger effective in preventing lipid peroxidation, did not inhibit EDB-induced liver DNA damage. In addition, unlike CCl_4, EDB induces slow sedimentation of liver DNA in alkaline sucrose gradients at non-necrogenic doses.

Slow sedimentation of DNA in alkaline sucrose gradients has been observed under other experimental conditions. Low doses of ionizing radiation releases DNA from a "complex" presumably consisting of lipid and protein in addition to DNA [39]; higher doses cause fragmentation of such released DNA [39]. The former phenomenon may be noticed when the mammalian cells are lysed for shorter time periods in alkali containing no detergents. In our experiments since sodium dodecyl sulfate was used in the alkaline lysing solution, the slow sedimentation of liver DNA induced by EDB may not be due to the release of DNA from the DNA–lipoprotein "complex."

The results presented in this paper clearly suggest that EDB induces *in vivo* both chemical (as measured by DNA-associated radioactivity) and physical (as measured by sedimentation analysis in alkaline sucrose gradients) lesions in liver DNA. In addition, EDB interacts with RNA and proteins (see Table 1) and characterization of these lesions may help in understanding the mutagenic and carcinogenic potential of this pesticide.

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REFERENCES

1. E. Nachtomi, E. Alumot and A. Bondi, *Israel J. Chem.* **6**, 803 (1968).
2. E. Nachtomi and E. Alumot, *Expl molec. Path.* **16**, 71 (1972).
3. A. Bondi, E. Olomucki and M. Calderon, *J. Sci. Fd Agric.* **6**, 600 (1955).
4. D. Amir, *J. Reprod. Fert.* **35**, 519 (1973).
5. W. A. Olson, R. T. Haberman, E. K. Weisburger, J. M. Ward and J. H. Weisburger, *J. natn. Cancer Inst.* **51**, 1993 (1973).
6. H. Brein, A. B. Stein and H. S. Rosenkranz, *Cancer Res.* **34**, 2576 (1974).
7. J. I. Goodman and V. R. Potter, *Cancer Res.* **32**, 766 (1972).
8. R. Cox, I. Damjanov, S. E. Abanobi and D. S. R. Sarma, *Cancer Res.* **33**, 2114 (1973).
9. I. Damjanov, R. Cox, D. S. R. Sarma and E. Farber, *Cancer Res.* **33**, 2122 (1973).
10. D. S. R. Sarma, R. O. Michael, B. W. Stewart, R. Cox and I. Damjanov, *Fedn Proc.* **32**, 833 (1973).
11. R. Cox, I. Damjanov and C. C. Irving, *Proc. Am. Ass. Cancer Res.* **14**, 28 (1973).
12. B. W. Stewart, S. Mirvish and E. Farber, *Biochem. biophys. Res. Commun.* **53**, 773 (1973).
13. B. W. Stewart and E. Farber, *Cancer Res.* **33**, 3209 (1973).
14. R. O. Michael, S. Parodi and D. S. R. Sarma, *Chem. Biol. Interact.* **11**, 19 (1975).
15. K. Kanagalingam and E. M. Balis, *Proc. Am. Ass. Cancer Res.* **15**, 46 (1974).
16. D. S. R. Sarma, J. Zubroff, R. O. Michael and S. Rajalakshmi, *Excerpta Medica int. Cong. Ser. No.* 350, **2**, 82 (1975).

17. N. L. R. Bucher, *Int. Rev. Cytol.* **15,** 245 (1963).
18. J. Zubroff and D. S. R. Sarma, *Analyt. Biochem.* **70,** 387 (1976).
19. S. Parodi, R. A. Mulivor, J. T. Martin, C. Nicolini, D. S. R. Sarma and E. Farber, *Biochim. biophys. Acta* **407,** 174 (1975).
20. K. S. Kirby and E. A. Cook, *Biochem. J.* **104,** 254 (1967).
21. W. C. Flamm, H. E. Bond and H. E. Burr, *Biochim. biophys. Acta* **129,** 310 (1966).
22. W. L. Schneider, *J. biol. Chem.* **161,** 293 (1945).
23. K. Burton, *Biochem. J.* **62,** 315 (1956).
24. W. Mejbaum, *Hoppe-Seyler's Z. physiol. Chem.* **258,** 117 (1939).
25. O. H. Lowry, A. L. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
26. P. Böhlen, S. Stein, W. Dairman and S. Udenfriend, *Archs Biochem. Biophys.* **155,** 213 (1973).
27. D. S. R. Sarma, S. Rajalakshmi and E. Farber, in *Cancer A Comprehensive Treatise* (Ed F. F. Becker), Vol. 1, p. 235. Plenum Publications, New York (1975).
28. H. Broda, E. Nachtomi and E. Alumot, *Comp. gen. Pharmac.* in press.
29. R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15,** 132 (1966).
30. L. M. Lutz, E. A. Glende and R. O. Recknagel, *Biochem. Pharmac.* **22,** 1729 (1973).
31. D. S. R. Sarma, S. Rajalakshmi and D. Hadjiolov, *Fedn Proc.* **33,** 595 (1974).
32. S. E. Abanobi, J. A. Popp, S. K. Chang, G. W. Harrington, P. D. Lotlikar, D. Hadjiolov, M. Levitt, S. Rajalakshmi and D. S. R. Sarma, *J. natn. Cancer Inst.* **58,** 263 (1977).
33. T. Sakaguchi, H. Nishimura, K. Masuda, I. Tusge, K. Onishi and H. Tutsami, *Biochem. Pharmac.* **15,** 756 (1966).
34. P. Czygan, H. Griem, A. J. Garro, E. Hutterer, F. Schaffner, H. Popper, O. Rosenthal and D. Y. Cooper, *Cancer Res.* **33,** 2983 (1973).
35. B. Stripp, F. E. Greene and J. R. Gillette, *J. Pharmac. exp. Ther.* **170,** 347 (1969).
36. E. Nachtomi, *Biochem. Pharmac.* **19,** 2833 (1970).
37. I. G. Walker and D. F. Ewart, *Mutation Res.* **19,** 331 (1973).
38. K. V. Shooter and R. K. Merrifield, *Chem. Biol. Interact.* **13,** 223 (1976).
39. M. M. Elkind and C. M. Chang Lu, *Int. J. Radiat. Biol.* **22,** 75 (1972).