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Ethylene dibromide: effects of paternal exposure on the neurotransmitter enzymes in the developing brain of F_1 progeny

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Summary

The effects of ethylene dibromide (EDB) exposure to male rats on several neurotransmitter enzymes have been examined in various brain regions of the F_1 progeny, from 7 to 90 days of age. The choline acetyltransferase activity was significantly increased at 21 days old, in most brain regions studied in the F_1 progeny of the EDB-treated males, but not at 7, 14 or 90 days old. The acetylcholinesterase activity was altered in different brain regions of the F_1 progeny of the EDB-exposed males at both 14 and 21 days old but not at 7 or 90 days old. Glutamic acid decarboxylase activity was increased in corpus striatum but decreased in frontal cortex only at 21 days of age. These neurochemical changes in the developing brain of F_1 progeny of EDB-treated males at low doses may be associated with behavioral abnormalities observed early in their development.

Halogenated hydrocarbons are one of the most important categories of industrial chemicals (Fishbein, 1976, 1980). Ethylene dibromide (EDB), a widely used bromide-based chemical, is used as a lead scavenger in gasoline (Fishbein, 1980) and as a fumigant in grain milling and the citrus industry (David, 1983).

EDB is shown to be mutagenic in a number of test systems including DNA modification (Brem et al., 1974), plant metabolic activation (Scott et al., 1978), various metabolizing systems such as glutathione in *Salmonella typhimurium* (Rannug et al., 1978; Rannug, 1980), hypoxanthine–guanine phosphoribosyl transferase (HGPRT) in the Chinese hamster ovary (CHO) (Tan and Hsie, 1981; Brimer et al., 1982), somatic mutation Tradescantia (Sparrow et al., 1974), and *Drosophila melanogaster* (Vogel and Chandler, 1974; Kale and Baum, 1983). EDB also affects spermatogenesis (Edwards et al., 1970; Amir, 1973; Courtens et al., 1980), and specific isozymes of cytochrome P-450 in rats (Moody et al., 1982). However, the effects of paternal exposure to EDB on the brain chemistry of F_1 progeny have not been explored. Significant behavioral abnormalities have been observed early in their development in the F_1 offspring of EDB-exposed male rats at dosages as low as 6.25 (1.25 mg/kg/day × 5 days) in the 4-week breeding treatment (Fanini et al., 1984). This dosage is considerably lower than the ones demonstrating dominant lethality (Edwards et al., 1970) or effects on sperm morphology (unpublished personal observations). These behavioral results

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TABLE 1

EFFECTS OF PATERNAL EXPOSURE TO EDB ON BRAIN ChAT ACTIVITY OF F_1 PROGENY

Brain regions	Day after birth							
	7		14		21		90	
	Control	EDB	Control	EDB	Control	EDB	Control	EDB
Cerebellum	6.56 ± 1.30	7.91 ± 0.89	8.75 ± 1.17	8.42 ± 0.97	8.18 ± 0.34	10.10 ± 0.44 *	7.23 ± 0.95	8.88 ± 0.84
Corpus striatum	6.06 ± 0.71	4.82 ± 0.51	8.77 ± 0.52	7.64 ± 0.64	14.53 ± 0.89	18.71 ± 1.19 *	27.67 ± 2.42	33.42 ± 3.40
Frontal cortex	8.06 ± 0.98	6.87 ± 0.56	8.03 ± 0.75	7.89 ± 0.65	21.49 ± 0.80	23.36 ± 0.82	10.44 ± 0.86	11.27 ± 0.37
Hippocampus	9.11 ± 1.35	12.50 ± 0.71	15.01 ± 0.68	12.89 ± 1.39	22.66 ± 1.25	32.79 ± 1.21 *	14.75 ± 0.86	15.00 ± 1.24
Hypothalamus	5.36 ± 0.73	3.85 ± 0.36 **	5.63 ± 1.00	7.19 ± 0.78	9.59 ± 1.18	12.25 ± 2.21 *	7.26 ± 0.92	6.31 ± 1.60

ChAT activity is expressed as nmoles acetylcholine produced/mg protein/15 min. Each value represents the mean ± S.E. of 4–9 animals. Statistical analyses were performed by Mann–Whitney *U* test.

* $p < 0.01$ vs. control.

** $p < 0.05$ vs. control.

TABLE 2

EFFECTS OF PATERNAL EXPOSURE TO EDB ON BRAIN AChE ACTIVITY OF F_1 PROGENY

Brain regions	Days after birth							
	7		14		21		90	
	Control	EDB	Control	EDB	Control	EDB	Control	EDB
Cerebellum	0.533 ± 0.068	0.527 ± 0.060	0.669 ± 0.037	0.577 ± 0.023 *	0.511 ± 0.064	0.292 ± 0.003 *	0.463 ± 0.05	0.505 ± 0.148
Corpus striatum	1.678 ± 0.088	2.298 ± 0.194 *	3.174 ± 0.267	3.112 ± 0.070 *	2.083 ± 0.095	2.042 ± 0.073	1.273 ± 0.168	1.323 ± 0.058
Frontal cortex	1.277 ± 0.046	1.189 ± 0.062	1.469 ± 0.048	1.520 ± 0.099	1.417 ± 0.063	1.317 ± 0.056	0.570 ± 0.042	0.529 ± 0.088
Hippocampus	0.762 ± 0.067	0.982 ± 0.030 *	1.144 ± 0.041	0.939 ± 0.038 *	0.259 ± 0.022	0.338 ± 0.019 *	0.873 ± 0.053	0.966 ± 0.041
Hypothalamus	0.522 ± 0.042	0.552 ± 0.052	0.668 ± 0.026	0.608 ± 0.053	0.601 ± 0.040	0.803 ± 0.034 *	0.874 ± 0.068	0.664 ± 0.082

AChE activity is expressed as moles acetylthiocholine hydrolyzed/mg protein/min. Each value represents mean ± S.E. of 4–9 animals. Statistical analyses were performed by Mann–Whitney *U* test.

* $p < 0.05$ vs. control.

prompted us to examine the possible biochemical changes in the developing brain of F_1 generation offspring of EDB-exposed males. In this paper we present data on the effects of paternal exposure to EDB on several neurotransmitter enzymes from various regions of the developing brain of F_1 progeny. The enzymes studied included choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and glutamic acid decarboxylase (GAD).

Materials and methods

[1-^{14}C]Acetyl-coenzyme A ([1-^{14}C]AcCoA, spec. act. 57 mCi/mmole) was purchased from Amersham Searle, Arlington Heights, IL and L-[U-^{14}C]glutamic acid (spec. act. 293 mCi/mmole) was obtained from New England Nuclear, Boston, MA. Choline chloride, acetylthiocholine, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), cetyltrimethylammonium bromide, Saponin, sodium glutamate, and pyridoxyl phosphate were obtained from Sigma Chemical Company, St. Louis, MO. Dowex 1-X8 anion exchange resin (chloride form, 200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA. Scintiverse counting solution was obtained from Fisher Scientific Company. Other chemicals were from standard sources with optimal purity. EDB was obtained from Aldrich Chemical Company, with a purity of 99%.

Young adult Fisher 344 strain male rats (90–120 days old) derived from the breeding colony in our laboratory were used. The male rats were treated with a daily dosage of EDB (1 mg/kg/day) on a subacute i.p. schedule for 5 successive days, corn oil was used as a vehicle and the solutions were prepared daily. The amount of fluid administered was 0.1 ml/100 g of body weight. Groups of males that received a 5-day i.p. dose of corn oil (0.1 ml/100 g) served as a vehicle control. Beginning 7 days after the last injection, the EDB-treated and the corn-oil-treated males were crossed with untreated virgin females (2 : 1 female : male ratio). The breeding continued for 7 days. Successful mating was indicated by the presence of a vaginal sperm plug (day 0 of pregnancy). The F_1 progeny at 7, 14, 21 and 90 days of age were sacrificed by decapitation. Brains were rapidly removed, selected regions including cerebellum, corpus striatum, frontal cortex, hippocampus and hypothalamus were dissected out on ice and stored at −60°C until assayed. Since in the newborns there were no significant differences in the brain enzyme activities between males and females both sexes at 7, 14 and 21 days old were combined for the study. For the 90-day-old animals, only males were used for the enzyme assays.

Enzyme assays

At the end of each experimental treatment, frozen tissues from rat brain regions were homogenized in 5 vol. of ice-cold 0.05 M potassium phosphate buffer, pH 7.2, and aliquots of the homogenates were used for ChAT, AChE and GAD assays. Protein concentration in each homogenate was determined by the method of Lowry et al. (1951).

ChAT activity in each homogenate was determined by the method of Schrier and Shuster (1967) with slight modifications. The enzyme activity in each brain tissue homogenate (0.1–0.2 mg protein) was measured by the rate of conversion of choline to [^{14}C]acetylcholine in the presence of 0.3 M NaCl, 0.1 mM physostigmine, 6 mM choline chloride, and 0.02 mM [^{14}C]AcCoA, at 37°C. Dowex Ag 1–8 × was used to adsorb the unreacted [^{14}C]AcCoA. The radioactivity of [^{14}C]ACh was measured in a Beckman LS-350 scintillation spectrometer. Duplicate determinations were performed for each homogenate.

AChE activity in each tissue homogenate was determined by the method of Garry and Routh (1965) with modifications. The reaction mixture containing 2 ml of 0.5 M Tris–HCl buffer, pH 7.4, 0.01% 5,5′-dithiobis(2-nitrobenzioc acid) (DTNB) was preincubated at 37°C for 5 min. An aliquot of 0.01 ml of Saponin (1%)-treated homogenate (1 : 2) and 0.25 ml of substrate (acetylthiocholine) were added to the reaction mixture which was allowed to be incubated at 37°C for exactly 15 min. At the end of each incubation, 0.5 ml of 0.1% cetyltrimethylammonium bromide (inhibitor) was added to the reaction mixture to stop the reaction. The absorbance of the final reaction mixture was measured immediately in a Gilford UV-visible spectrophotometer at 415 nm with a 10 mm curvette. Reaction mixtures with 0.5 ml of inhibitor added prior to the addition of acetylthiocholine served as blanks. The AChE activity is expressed as moles of

TABLE 3

EFFECTS OF PATERNAL EXPOSURE TO EDB ON BRAIN GAD ACTIVITY OF F_1 PROGENY

Brain regions	Days after birth							
	7		14		21		90	
	Control	EDB	Control	EDB	Control	EDB	Control	EDB
Cerebellum	0.07 ± 0.010	0.077 ± 0.015	0.088 ± 0.001	0.083 ± 0.003	0.071 ± 0.002	0.083 ± 0.005	0.109 ± 0.003	0.133 ± 0.024
Corpus striatum	0.015 ± 0.002	0.013 ± 0.001	0.041 ± 0.001	0.038 ± 0.002	0.037 ± 0.044	0.064 ± 0.006 *	1.301 ± 0.090	1.252 ± 0.030
Frontal cortex			0.022 ± 0.001	0.018 ± 0.002	0.151 ± 0.006	0.136 ± 0.006 *	0.187 ± 0.007	0.164 ± 0.006 *
Hippocampus					0.133 ± 0.004	0.117 ± 0.006	1.503 ± 0.062	1.080 ± 0.068
Hypothalamus					0.115 ± 0.003	0.079 ± 0.011	0.169 ± 0.013	0.115 ± 0.021

GAD activity is expressed as nmoles CO_2 produced/mg protein/h. Each value represents the mean ± S.E. of 4–9 animals. Statistical analyses were performed by Mann–Whitney *U* test.

* $p < 0.05$ vs. control.

substrate hydrolyzed/min/mg protein based on molar extinction coefficient of 13600.

GAD activity in each tissue homogenate was determined by the method of Wu et al. (1978) with some modifications, measuring the $^{14}CO_2\uparrow$ produced from [^{14}C]Na-glutamate catalyzed by the enzyme. The $^{14}CO_2\uparrow$ was trapped in 0.2 ml of hyamine and radioactivity was measured in a toluene-based counting solution. Duplicate determinations were performed for each sample.

Results

Effects of paternal exposure to EDB on regional brain ChAT activity in the developing brain

EDB-exposed adult male rats mated at 7–14 days following treatment resulted in significant changes in ChAT in various brain regions of F_1 progeny at 21 days after birth but not at 7, 14 or 90 days after birth (Table 1). In 21-day-old F_1 progeny the specific ChAT activity was significantly increased in cerebellum by 25%, corpus striatum by 29%, hippocampus by 45% and hypothalamus by 28%. A slight although not significant increase in ChAT activity was also observed in frontal cortex at 21 days of age.

Effects of paternal exposure to EDB on regional AChE activity in the developing brain of F_1 progeny

The specific AChE activity was altered in various brain regions of F_1 progeny at 7, 14 and 21 days after birth but not affected at all at 90 days after birth (Table 2). At 7 days after birth in the F_1 progeny from EDB-treated males, AChE activity was increased in corpus striatum by 37% and increased in hippocampus by 29% but was not affected in cerebellum, frontal cortex or hypothalamus. At 14 days after birth, the specific AChE activity was significantly decreased in the cerebellum by 14%, corpus striatum by 16%, and hippocampus by 18% but not changed in the other brain regions examined of the F_1 progeny from EDB-treated males.

At 21 days after birth, the specific AChE activity was decreased by 43% in cerebellum, increased in hippocampus by 31% and in hypothalamus by 34% and was not affected in corpus striatum and frontal cortex. Lastly, at 90 days after birth, the specific AChE activity was not altered in any of the 5 brain areas studied in the F_1 progeny of EDB-treated males.

Effects of paternal exposure to EDB on regional GAD activity in the developing brain of F_1 progeny

Specific GAD activity was altered in various brain areas of F_1 progeny from EDB-treated males only at 21 and 90 days after birth but not at 7 or 14 days after birth (Table 3). At 21 days after birth, the GAD activity was significantly increased in corpus striatum by 79% whereas it was significantly decreased in frontal cortex by 10%; and this enzyme activity was not affected in cerebellum, hippocampus or hypothalamus. At 90 days after birth, the GAD activity was significantly decreased in frontal cortex by 12% but was not affected in any other areas examined.

Discussion

In this study, the exposure of adult male rats to a low dosage of EDB induced significant increases in ChAT in various brain regions of F_1 progeny at 21 days of age (Table 1), but did not affect this enzyme activity in F_1 progeny at other ages studied. The paternal EDB-induced increases in ChAT were observed to be highest in hippocampus (by 45%), followed in descending order by corpus striatum (by 29%), hypothalamus (by 28%), and cerebellum (by 25%). However, no significant changes were observed in the frontal cortex of F_1 progeny at 21 days of age. These results indicated that the cholinergic systems in the F_1 progeny were perturbed at the age of 21 days in most brain regions examined. These disturbances in ChAT activity seemed to have been recovered by 90 days of age when the enzyme activity declined to the normal levels in all the 5 brain regions studied.

In the F_1 progeny, the brain AChE activity was also altered in cerebellum, corpus striatum, hippocampus and hypothalamus but not in frontal cortex, at 7, 14 and 21 days of age. However, the changes in AChE activities were biphasic, depending on the age of the animals and the brain regions examined. For example, at 7 days old in both corpus striatum and hippocampus the AChE activity was elevated by 37% and 29% respectively, in the F_1 progeny of EDB-treated males compared to the controls. At 14 days of age this enzyme activity

was slightly but significantly inhibited in these two brain areas (16% and 18% respectively). On the other hand, at 21 days of age, AChE was markedly decreased in cerebellum (43%) but increased in hippocampus (31%) and hypothalamus (34%) in the offspring of EDB-treated male rats. Thus, it would appear difficult to consider these biphasic changes in AChE purely as genetically triggered abnormalities. There is a possibility that different forms of AChE in the brain (Hsu, 1982; Rieger and Vigny, 1976) might have been affected by the paternal exposure to EDB and hence resulted in altered developmental patterns of AChE activity.

At 90 days, again no differences in AChE activity were observed in any of the brain regions studied. It seemed, therefore, that the disturbance in the cholinergic neurons of the offspring following paternal EDB treatment may indeed be recoverable as indicated above by the ChAT activity.

Lastly, concerning the GABAergic systems, at 21 days of age, the GAD activity was markedly increased in corpus striatum (by 79%) but slightly decreased in the frontal cortex (by 10%). By 90 days of age, the striatal GAD activity was comparable to that found in control animals. However, the decrease in GAD in the frontal cortex persisted into adulthood (Table 3), indicating a possible permanent damage of the GABAergic neurons at least in the frontal cortex after paternal EDB exposure.

These are the first pieces of information with regard to the postmeiotic effects of paternal exposure to EDB on the brain chemistry of F_1 progeny. Our results clearly indicated that both cholinergic and GABAergic neuronal systems in specific brain areas were perturbed during development. These changes in the brain neurotransmitter systems may well be associated with the behavioral abnormalities observed in the F_1 progeny of EDB-exposed male rats (Fanini et al., 1984).

The present findings are based upon a random selection of F_1 offspring from the litters derived from the EDB-exposed male rats. If whole litters were utilized in the brain chemistry assays one might observe a greater variability in the offspring of the EDB-exposed males due to the randomness of the mutations induced. However, it is difficult to assess the extent to which the F_1 offspring are affected without individual cytogenetic assessments and F_2 generation breeding studies.

At present, the exact mechanism of the male-mediated effects of EDB on the brain chemistry of F_1 progeny is unclear. However, studies of the intrinsic properties including kinetic parameters, K_m and V_{max} values, of these brain neurotransmitter enzymes would be important to further define the mechanism of EDB effects. Furthermore, determination of the amino acid sequences of these brain enzymes in F_1 progeny of the EDB-treated animals will shed light on the possible structural mutation of these enzymes induced by the paternal EDB exposure. As alkyl bromides react readily with thiols, amines and other nucleophilic biochemical compounds (Ross, 1962; Kendorosi et al., 1973; Gould, 1959), and may form covalent bonds with biological substances under physiological conditions (Meneghini, 1974; Vogel and Chandler, 1974), it would be conceivable that certain DNA or RNA components in the EDB-treated animals might be chemically modified by EDB and thus result in enzyme mutation in the F_1 progeny. If the enzyme mutation did occur in the F_1 progeny, such mutation should also be expressed in the F_2 generation. Therefore it would be important to examine these enzyme activities in the F_2 progeny. Such experiments are underway in our laboratory. The effects of EDB on the sperm DNA and RNA composition and sequence that regulate the syntheses of the brain enzymes remain to be elucidated.

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References

Amir, D. (1973) Sites of spermicidal action of ethylene dibromide in bulls, J. Reprod. Fert., 35, 519.

Brem, H., A.B. Stein and H.S. Rosenkranz (1974) The mutagenicity of DNA-modifying effect of haloalkanes, Cancer Res., 34, 2576–2579.

Brimer, P.A., E.L. Tan and A.W. Hsie (1982) Effects of metabolic activation on the cytotoxicity and mutagenicity of

1,2-dibromoethane in the CHO/HGPRT system, Mutation Res., 95, 377–388.

Courtens, J.-L., D. Amire and J. Durand (1980) Abnormal spermiogenesis in bulls treated with ethylene dibromide: An ultrastructural and ultracytochemical study, J. Ultrastruct. Res., 71. 103–115.

David, P. (1983) Ethylene dibromide, Tighter rules on use proposed, Nature (London), 22–28, 263.

Edwards, K., H. Jackson and A.R. Jones (1970) Studies with alkylating agents, II. A chemical interpretation through metabolic studies of the antifertility effects of ethylene dimethanesulphonate and ethylene dibromide, Biochem. Pharmacol., 19, 1783.

Fanini, D., M.S. Legator and P.M. Adams (1984) Effects of paternal ethylene dibromide exposure on F_1 generation behavior in the rat, Mutation Res., 139, 133–138.

Fishbein, L. (1976) Industrial mutagens and potential mutagens, I. Halogenated aliphatic derivatives, Mutation Res., 32, 267–308.

Fishbein, L. (1980) Production uses, and environmental fate of ethylene dichloride and ethylene dibromide, in: B. Ames, P. Infante and R. Reitz (Eds.), Banbury Report 5, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 227–238.

Garry, P.J., and J.I. Routh (1965) A micro method for serum cholinesterase, Clin. Chem., 11, 91–96.

Gould, E.S. (1959) Mechanism and Structure in Organic Chemistry, Holt, Rhinerhart and Wilson, New York, pp. 250–305, 561–611.

Hsu, L.L. (1982) Minireview: Brain arylacylamidase, Int. J. Biochem., 14, 1037–1042.

Kale, P.G., and J.W. Baum (1983) Sensitivity of *Drosophila melanogaster* to low concentration of gaseous mutagens, Mutation Res., 113, 135–143.

Kendorosi, A., T. Fedorcsah and F. Solymosy (1973) Inactivation of Q-beta RNA by electrophiles, Mutation Res., 17, 149–161.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent, J. Biol. Chem., 193, 265–275.

Meneghini, R. (1974) Repair replication of opossum lymphocyte DNA, effect of compounds that bind to DNA, Chem.-Biol. Interact., 8, 113–126.

Moody, D.E., G.A. Clawson, C.H. Woo and E.A. Smuckler (1982) Cellular distribution of cytochrome P-450 loss in rats of different ages treated with alkyl halides, Toxicol. Appl. Pharmacol., 66, 278–289.

Rannug, U. (1980) The use of different metabolizing systems in the elucidation of the mutagenic effects of ethylene dichloride in Salmonella, in: B. Ames, P. Infante and R. Reitz (Eds.), Banbury Report 5, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 83–92.

Rannug, U., A. Sundvall and C. Ramel (1978) The mutagenic effect of 1,2-dichloroethane on *Salmonella typhimurium*, I. Activation through conjugation with glutathione in vitro, Chem.-Biol. Interact., 20, 1–16.

Rieger, R., and M. Vigny (1976) Solubilization and physicochemical characterization of rat brain acetylcholinesterase: Development and maturation of its molecular forms, J. Neurochem., 27, 121–129.

Ross, W.C.J. (1962) Relative reactivity of different nucleophilic centres towards alkylating agents, in: R.W. Raven (Ed.), Cancer Monograph Series, Butterworths, London. pp. 19–31.

Schrier, B.K., and L. Shuster (1967) A simplified radio-chemical assay for choline acetyltransferase, J. Neurochem., 14, 977–985.

Scott, B.R., A.H. Sparrow, S.S. Schwemmer and L.A. Schairer (1978) Plant metabolic activation of 1,2-dibromethane (EDB) to a mutagen of greater potency, Mutation Res., 49, 203–212.

Sparrow, A.H., L.A. Schairer and R. Villalobos-Pietrini (1974) Comparison of somatic mutation rates indiced in Tradescantia by chemical and physical mutagens, Mutation Res., 26, 265–276.

Tan, E.-L., and A.W. Hsie (1981) Mutagenicity and cytotoxicity of haloethanes as studies in the CHO/HGPRT system, Mutation Res., 90, 183–191.

Vogel, E., and J.L.R. Chandler (1974) Mutagenicity testing of cyclamate and some pesticides in *Drosophila melanogaster*, Experientia, 30, 621–623.

Wu, J.Y., T. Matsuda and E. Roberts (1978) Purification and characterization of glutamate decarboxylase from mouse brain, J. Biol. Chem., 248, 3029–3034.