Toxicity of Phthalate Esters to Nervous Tissue in Culture

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Phthalate ester plasticizers are known to have polluted our environment(MAYER et al.,1972;JAEGER and RUBIN,1970a;MORRIS,1970;OGNER and SCHNITZER,1970) and accumulated in human tissues(JAEGER and RUBIN,1970b). Toxic effects of these chemicals on some aquatic invertebrates(SANDERS et al.,1973), rats, rabbits, and humans(MALLETTE and VON HAAM,1952), fishes(MAYER et al., 1972), mice and rabbits(CALLEY et al.,1966), and humans (NEERGAARD et al.,1971) have been reported. Since phthalate esters have the hydrophobic property, the neurotoxic action of the compounds should be tested. However, the studies on the neurotoxicity of phthalate esters are limited. This paper reports the results of the toxicity of di-n-butyl phthalate(DNBP), diethyl phthalate(DEP), and dimethyl phthalate(DMP) on cerebellar tissue from newborn rats in tissue culture, and comparative studies on the relationship between chemical properties and the toxicities of these phthalate esters.

Methods

Cerebella from newborn rats (6-12 days old) were sliced into small particles of 1-2 mm^3, and cultured on collagen-coated plastic dishes(Falcon dishes for cell cultures, 35 X 10 mm) at 36.5° C for 4 days in a CO_2 incubator. The culture medium contained calf serum and Eagle MEM(1:1)(did not contain L-glutamine). Glucose was added to the medium at a final concentration of 550 mg/100 ml. Phthalate esters were dissolved in calf serum directly(each 1 µl/ml serum) and appropriate amounts of the serum were added to the medium to bring about the desired concentrations of the phthalate esters.

The degree of outgrowth was expressed in two ways. In Table I, it was expressed as a function of the outgrowth of nerve fibers and fibroblasts considering the degenerative changes as follows.

VI:Outgrowth of nerve fibers and fibroblasts.
V:Normal outgrowth of fibers and depressed outgrowth of fibroblasts, or depressed outgrowth of fibers and normal outgrowth of fibroblasts.
IV:Depressed outgrowth of fibers and fibroblasts.
III:No outgrowth of fibers and normal outgrowth of

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fibroblasts, or normal outgrowth of fibers and no outgrowth of fibroblasts.

II: Depressed outgrowth of fibers and no outgrowth of fibroblasts, or no outgrowth of fibers and depressed outgrowth of fibroblasts.

I: No outgrowth of fibers and fibroblasts.

In Fig. 1, the degree of outgrowth was expressed by the number of explants that showed outgrowth of nerve fibers without considering either depressed outgrowth or degenerative changes of the fibers.

Results

The toxic effects of phthalate esters on cerebellar tissue in culture are shown in Table I.

At a concentration of 3.1 X 10^{-4}M DMP, some explants showed depressed network formation of nerve fibers; i.e., the fibers extended straight and often were partially torn from the surface of the dish. Glial cells and fibroblasts showed normal outgrowth. Explants that showed depressed network formation of the fibers increased at 6.1 X 10^{-4}M DMP and the depressed network portion often showed curvature. Fibroblasts developed forming a sheet. At 18.3 and 30.5 X 10^{-4}M DMP, an incomplete inhibition of the outgrowth and/or depression of network formation of the fibers was observed. Depressed outgrowth of glial cells was observed accompanying depressed outgrowth of the fibers. Fibroblasts showed almost normal outgrowth at these high concentrations of DMP, although dispersed and atrophic portions were observed in part.

At a concentration of 2.6 X 10^{-4}M DEP, the sheet-like development of fibroblasts was depressed, but nerve fibers and glial cells showed almost normal outgrowth. In the presence of 5.1 X 10^{-4}M DEP, some explants showed no development of nerve fibers, and no sheet formation of fibroblasts. At concentrations of 15.3 and 25.5 X 10^{-4}M DEP, the outgrowth of nerve fibers was strongly depressed and no development of the fibers was found on some explants. Degenerative change of the fibers such as granulation was not apparent. Glial cells showed development in the explants which showed no outgrowth of the fibers. Fibroblasts showed no sheet formation and developed dispersedly, and in some explants no outgrowth of fibroblasts was observed. Degenerative changes such as granulation and deformation of the spindle form of fibroblasts were not obvious although slight atrophic change was observed.

In the presence of 2.0 X 10^{-4}M DNBP, no detectable changes were observed in nerve fibers, glial cells, and fibroblasts. At a concentration of 3.9 X 10^{-4}M DNBP, the fibers showed granulation and the outgrowth of fibroblasts was depressed. The outgrowth of nerve fibers was very depressed and degenerative changes such as granulation and lytic change were obvious at the concentration

TABLE 1

The effects of phthalate esters on nerve fibers and fibroblasts from newborn rat cerebellum in tissue culture.

		Phthalate esters ($\times 10^{-4}$ M)															
		DMP					DEP					DNBP					
		0	3.1	6.1	18.3	30.5	0	2.6	5.1	15.3	25.5	0	2.0	3.9	7.8	11.7	19.5
Degree of outgrowth	VI	8	5	3	1	2	9	4	4	2		9	7	2			
	V	2	5	6	8	6	1	3	2	2	1	1	3	3	4		
	IV			1	1	1			1		1			3	2		
	III							3	1	1	1			1	2	1	
	II					1			2	3	6			1	2	8	8
	I									2	1					1	2
U-values[a]			35[b]	24	14	18		23.5	23	12	0.5		40[b]	12.5	2	0	0

[a]The Mann-Whitney U test. Critical values of U for a two-tailed test at α =0.05 and 0.10 are 23 and 27, respectively.

[b]Not significantly different from control.

Numerals in the Table show the number of explants of cerebellum. On the degree of the outgrowth, see text.

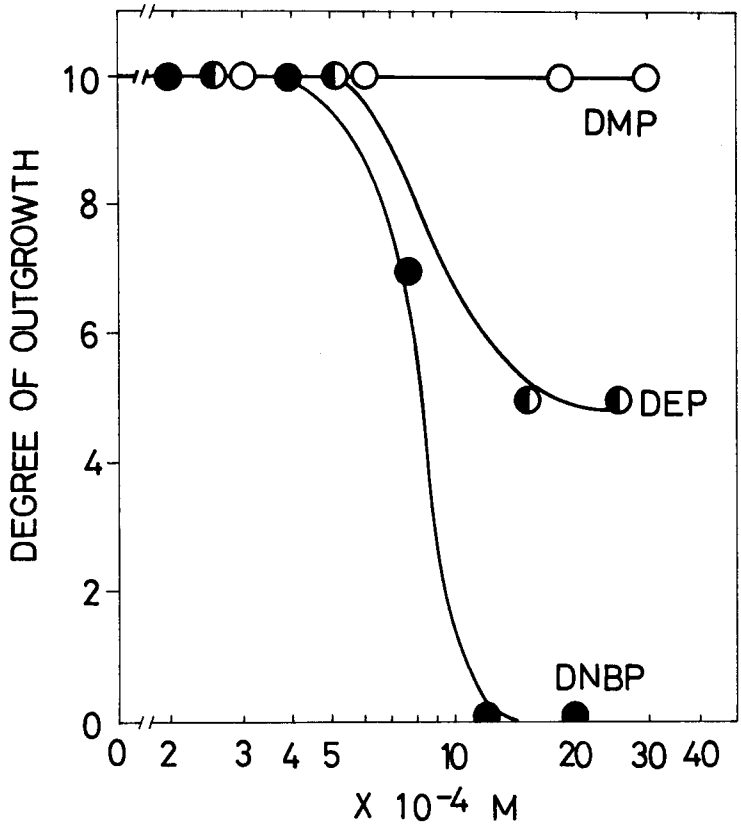

Fig. 1. Toxic effects of DMP, DEP, and DNBP on nerve fibers from cerebellum in culture. Degree of outgrowth:(explants showed the outgrowth of nerve fibers/explants served for culture) X 10.

of 7.8 X 10^{-4} M. The development of glial cells was also depressed at this concentration. Furthermore, granules in the fibroblasts and atrophy of the cells were observed with depressed sheet formation. At the high concentrations of 11.7 and 19.5 X 10^{-4} M, the fibers and glial cells did not show any outgrowth, whereas a few fibroblasts showed migration accompanied by granules and deformation of the cells.

Toxicities of DMP, DEP, and DNBP on nerve fibers are depicted in Fig. 1. All explants showed outgrowth of the fibers at a concentration of 30.5 X 10^{-4} M DMP. No explants of the cerebellar tissue showed any outgrowth of nerve fibers at a concentration of 11.7 X 10^{-4} M DNBP, while half of the explants showed some outgrowth of the

fibers at a concentration of 15.3×10^{-4} M DEP.

From these results, the order of toxicity of these three phthalate esters was determined as follows:

DNBP > DEP > DMP

Discussion

MALLETTE and VON HAAM(1952) reported that butyl-benzyl phthalate killed rats after intraperitoneal administration of doses higher than 1.8 g or after oral administration of more than 4 g/kg of body weight, and produced degenerative lesions of the central nervous system with congestive encephalopathy, myelin degeneration, and glial proliferation. Furthermore, CALLEY et al.(1966) reported that DMP, DEP, and dibutyl phthalate appeared to demonstrate central nervous system depression, and the electroencephalogram depression pattern was obtained after intravenous administration of di-(methoxy-ethyl) phthalate to rabbits. However, pathological changes of central nervous system due to DNBP, DEP, and DMP are not apparent. As reported in this paper, it may be concluded that DNBP, DEP, and DMP are able to inhibit outgrowth of the nervous system and produce degenerative changes in the tissues. It is notable that nerve fibers are more sensitive than fibroblasts to DNBP and DMP.

It was reported that the toxicity of phthalate esters was parallel to their water solubility(greater solubility, greater activity) and inversely parallel to their molecular weight(lower molecular weight, greater activity), based on the results of LD_{50} estimation, intradermal irritation tests,and tissue culture toxicity studies on chick embryo cells and mouse fibroblasts (CALLEY et al., 1966). However, the results on nerve fibers presented in this paper indicate the reverse order; i.e., lower solubility, greater toxicity, and greater molecular weight, greater activity. The difference is probably not due to the difference of tissues used in the tests, since the same order of toxicity as the result of nerve fibers was obtained on fibroblasts from cerebellar tissue in culture. It may be reasonable to consider that solubility in water is the more important factor for absorption and distribution of the chemicals, whereas the hydrophobic property of the esters is more dominant in producing toxic action, at the stage of interaction of the esters with cells. The result on mouse fibroblasts(CALLEY et al., 1966) was obtained by placing porous pads wetted with 0.05 ml of a 50 mg/ml emulsion of phthalates on the surface of the agar. Under this experimental condition, the esters must dissolve in water before the reaction with the cells in culture.

The phthalate esters that have simple structure were

tested in this paper. However, more detailed experiments of the toxicological effects of other phthalate esters is essential to elucidate their neurotoxicity.

Summary

DNBP markedly inhibited the outgrowth of nervous tissue and produced degenerative changes at 7.8 X 10^{-4}M, and the minimal dose for total inhibition of nerve fibers and glial cells is at about 11.7 X 10^{-4}M. DEP affected the outgrowth of cerebellar tissue in culture at a concentration of 2.6 X 10^{-4}M, and strongly inhibited the outgrowth at 15.3 X 10^{-4}M. However, complete inhibition of the outgrowth by DEP was not observed even at 25.5 X 10^{-4}M. Degenerative changes of these fibers and cells were not apparent. Nerve fibers seemed to be more sensitive than glial cells to DEP. DMP had an adverse effect on cerebella in tissue cultures especially on nerve fibers, but it did not completely inhibit the outgrowth of the cells and fibers even at the concentration of 30.5 X 10^{-4}M. Nerve fibers and glial cells were more sensitive than fibroblasts to DNBP and DMP.

From these results, it was concluded that phthalate ester, which has a large molecular weight and low solubility in water, showed a high degree of toxicity.

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