

# Studies on the Genetic Effects of Phthalic Acid Esters on Cells in Culture

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Mono(2-ethylhexyl) phthalate (MEHP) induced chromosome aberrations in cells of two culture lines, one derived from Chinese hamster ovary cells (CHO) and the other from rat liver cells (RL4). In CHO cells, the clastogenicity of MEHP was unaffected by the presence of an exogenous metabolic activation system (S-9 mix). 2-Ethylhexanol, *o*-phthalic acid, and phthalic anhydride were without effect.

Cytochemical methods and assays for carnitine acetyltransferase and KCN-insensitive palmitoyl CoA oxidation were employed to determine whether chromosome damage was associated with peroxisome proliferation. No evidence of an increase in peroxisome numbers or of induction of marker enzymes was found in CHO cells treated with MEHP for up to 72 hr. Clofibrate and BR931 were also ineffective.

Observations on changes in CHO cell structure and permeability, and on the haemolytic effects of phthalate monoesters, suggest that the cytotoxicity of MEHP may be due primarily to its action on cell membranes. Since chromosome damage was observed only at cytotoxic concentrations, it is suggested that damage to lysosomal membranes and the release of endonucleases may be responsible for the observed clastogenicity of MEHP *in vitro*.

## Introduction

The National Toxicology Program study showed that continuous exposure of both rats and mice to a maximum tolerated dose of di(2-ethylhexyl) phthalate (DEHP) in the diet for 2 years resulted in an increase in hepatocellular carcinoma in both species (1). However, despite the deployment of a wide variety of short term test systems in numerous laboratories, there is little convincing evidence that DEHP or its metabolites is carcinogenic by virtue of a genotoxic mechanism. Against an extensive background of negative results with DEHP and MEHP in both bacterial and mammalian systems, there are reports of positive dominant lethal assays (2,3) and mutagenic and clastogenic effects in a wide-ranging study in one laboratory (4). Our own studies (5) have revealed a clastogenic effect of MEHP, but not DEHP, in Chinese hamster ovary (CHO) cells.

A possible explanation for the lack of effect of MEHP or DEHP in many mutagenicity test systems is provided by the hypothesis (6) that the carcinogenicity of DEHP and other hypolipidemic agents might be a result of genetic damage caused by hydrogen peroxide, generated by enzymes induced during peroxisome proliferation. We have shown that H<sub>2</sub>O<sub>2</sub>, generated exogenously by xanthine and xanthine oxidase, can cause genetic damage in CHO cells (7). Thus, it was of interest to determine whether the clastogenic effect of MEHP in these cells

was associated with peroxisome proliferation. However, no evidence of peroxisome proliferation was found. Various observations are presented that suggest that MEHP may be toxic to cultured cells by virtue primarily of its surface activity. The hypothesis proposed is that the observed chromosome changes are the result of lysosomal enzyme release within MEHP damaged CHO cells. The hemolytic effects of MEHP and some linear phthalate monoesters on rat erythrocytes are compared.

## Materials and Methods

*o*-Phthalic acid monoesters were synthesized by the method of Albro (8). Neutral solutions were prepared in phosphate buffered saline, by titration with NaOH, immediately before addition to culture medium.

Chinese hamster ovary cells of clone CHO-K1-BH<sub>4</sub> (9) were obtained from J. P. O'Neill (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) and maintained in Ham's F12 medium supplemented with 5% fetal calf serum. RL<sub>4</sub> cells (10) were supplied by B. J. Dean (Shell Research Ltd., Sittingbourne, Kent, U.K.). They were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

S9 mix was prepared as described by Ames et al. (11) by using liver from Aroclor-treated rats.

Chromosome analysis was carried out as described previously (5). Both CHO and RL<sub>4</sub> cells were exposed for 2 hr to medium containing MEHP and treated with colcemid (0.1 µg/mL) from 22 to 24 hr after the start of

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treatment. Metaphase arrested cells were prepared for chromosome analysis, and for each treatment 100 metaphases were scored for all types of aberration.

Assays for carnitine acetyltransferase and KCN insensitive palmitoyl CoA oxidation were carried out as described by Gray et al. (12). Cells were removed from treated cultures by scraping into Tris/HCl buffer (pH 7.4) and sonicated.

Hemolysis assays were performed using freshly isolated rat erythrocytes, washed twice with phosphate-buffered saline (pH 7.4) and suspended at a final concentration of  $10^9$  cells/mL in the same buffer containing the appropriate concentration of phthalate monoester. After incubation for 2 hr at 37°C, intact cells were removed by centrifugation and the amount of hemoglobin released was estimated by reading the absorbance at 540 nm after dilution of the supernatant with 3 volumes of 0.6% ammonium hydroxide.

## Results

### Chromosome Damage

MEHP treatment for 2 hr caused extensive chromosome damage in CHO cells (Table 1). A dose-related increase in aberrations including gaps, breaks, and chromatid exchanges was induced over a narrow range of concentrations; 1.5 mM was too toxic for chromosome aberrations to be scored. The presence of S-9 mix slightly reduced the total number of aberrations produced.

With RL<sub>4</sub> cells (Table 2), chromosome analysis was possible after treatment with higher concentrations of MEHP since growth inhibition was not seen below 6.5 mM. A dose-related increase in aberrations was found, and also an increase in endoreduplication.

To test whether the difference in sensitivity between CHO and RL<sub>4</sub> cells was an intrinsic property of the two cell lines or an effect of the use of two different culture media, CHO cells were treated with MEHP in either Ham's F12 or Dulbecco's medium. In each case, the normal growth medium (Ham's F12) was replaced after the 2-hr treatment. As shown in Table 3, MEHP was considerably less toxic to CHO cells when dissolved in Dulbecco's medium. Chromosome aberrations were only observed at MEHP concentrations just below those which caused complete growth inhibition.

### Peroxisomal Enzyme Assays

CHO cultures were treated with various concentrations of MEHP, clofibrate acid or BR931, either for 72 hr with addition of fresh compound every 24 hr or for 2 hr only as in the chromosome aberration tests. The concentration ranges employed included at least one concentration that was toxic, as shown by a large decrease in the total protein per culture. Assays for carnitine acetyltransferase and KCN-insensitive palmitoyl CoA oxidation revealed no induction of either enzyme after treatment with any of the compounds tested.

No evidence of increased numbers of peroxisomes was obtained by cytochemical methods applied to similar cultures.

### Cell Membrane Damage

When MEHP was added to the medium of CHO cells (Ham's F12) various cellular changes were observed. At low concentrations (below 0.5 mM), the cells became more rounded but did not degenerate. At higher concentrations, progressive degeneration occurred, culminating in disintegration of the cells. The rate of cell degeneration was concentration dependent and, above 2 mM MEHP lysis was almost instantaneous. These effects were very similar to those observed after treatment with sodium dodecyl sulfate (SDS), although SDS damage occurred more rapidly.

The ability of CHO cells to exclude Trypan Blue was assessed at various times after addition of MEHP. Dye uptake occurred prior to cell lysis and it was shown using duplicate cultures that cells recovered the ability to exclude Trypan Blue if MEHP was removed before the cells ruptured. Figure 1 shows the relationship between Trypan Blue uptake at the end of the 2-hr treatment with MEHP and the ability of MEHP-treated cells to grow into colonies. Also shown is the number of cells with chromosome aberrations 24 hr after treatment.

Assays using rat red blood cells clearly demonstrated the hemolytic activity of MEHP. Figure 2 shows the results obtained with MEHP and a series of straight-chain phthalate monoesters from *n*-butyl to *n*-octyl phthalate. Lysis occurred with 1 to 2 mM MEHP which was intermediate in effectiveness between the *n*-heptyl and *n*-octyl esters. Visual observations on CHO cells (not shown) gave

Table 1. Chromosome aberrations in CHO cells treated with MEHP for 2 hr in the presence or absence of S-9 mix.

MEHP concn, mM	S-9 mix	Aberrant cells, %	Aberrations per 100 cells			
			Total	Gaps <sup>a</sup>	Breaks <sup>a</sup>	Exchanges <sup>a</sup>
0	—	12	12	6	4	2
0	+	12	12	8	4	0
0.7	—	12	14	0	12	2
0.7	+	15	19	1	14	4
1.0	—	49	89	14	49	26
1.0	+	28	38	7	8	23
1.3	—	60	173	7	133	33
1.3	+	60	104	12	53	39

<sup>a</sup>Figures include aberrations of both the chromatid and chromosome type.

Table 2. Chromosome aberrations and endoreduplication in RL<sub>4</sub> cells treated with MEHP for 2 hr.

MEHP, concn. mM	Aberrant cells, %	Aberrations per 100 cells				Endoreduplication, %
		Total	Gaps <sup>a</sup>	Breaks <sup>a</sup>	Exchanges <sup>a</sup>	
0	2	2	0	2	0	0
2.0	9	9	1	7	1	4
3.0	10	14	0	12	2	12
4.0	16	25	1	16	8	24
5.0	26	42	1	31	10	10
6.5		No mitoses				

<sup>a</sup> Figures include aberrations of both the chromatid and chromosome type.

Table 3. Chromosome aberrations in CHO cells treated with MEHP in two culture media.

MEHP concn, mM	Aberrations per 100 cells	
	F12 medium	Dulbecco's medium
0	6	4
0.5	0	—
0.75	2	—
1.0	26	0
1.5	No mitoses	6
2.5	Cells lysed	4
5.0	—	52
10.0	—	No mitoses

similar results.

Cytochemical methods and enzyme assays demonstrated that acid phosphatase was released intracellularly and was later detectable in the culture medium after treatment of CHO cells with MEHP.

## Discussion

The clastogenic effect of MEHP on CHO cells, reported previously (5), was confirmed in the present study and demonstrated also in an unrelated cell line of rat liver origin (RL<sub>4</sub>). In RL<sub>4</sub> cells an increase in endoreduplication was also observed. Although the significance of endoreduplication is uncertain, it is one of the effects of

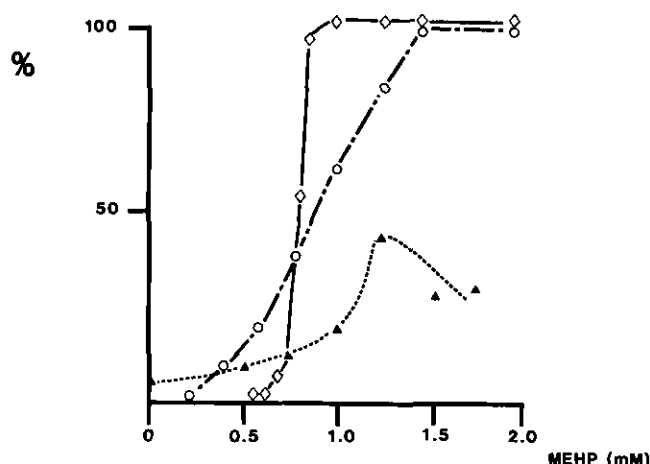


FIGURE 1. Relationship between (◇) percentage of CHO cells taking up Trypan Blue after 2 hr exposure to MEHP, (▲) percentage of cells showing chromosome aberrations after 24 hr, and (○) percent decrease in plating efficiency.

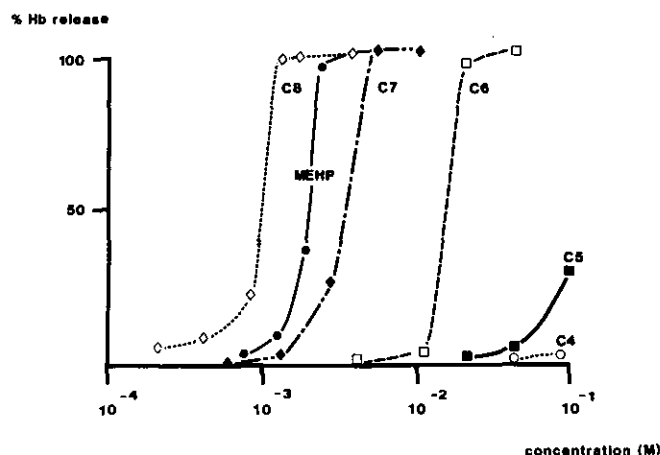


FIGURE 2. Release of hemoglobin from rat erythrocytes treated with MEHP, mono-*n*-butyl (C4), mono-*n*-pentyl (C5), mono-*n*-hexyl (C6), mono-*n*-heptyl (C7) or mono-*n*-octyl (C8) phthalate.

ionizing radiation (13) on cultured cells.

These results might be interpreted as a demonstration of the ability of MEHP to react with the DNA of mammalian cells. However, such a reaction seems unlikely on chemical grounds and has not been demonstrated *in vivo* or *in vitro* (14). In addition, negative results have been reported for MEHP in a variety of mutagenicity test systems, including the sister chromatid exchange tests and HGPRT cell mutation assay in CHO cells in our laboratory (5). The possibility that a clastogenic metabolite of MEHP is formed within CHO and RL<sub>4</sub> cells cannot be ruled out. However, S-9 mix, which activates a wide variety of mutagens, had no effect on the chromosome damage produced by MEHP in CHO cells. Negative results with 2-ethylhexanol and *o*-phthalic acid (5) and also phthalic anhydride (unpublished observations) suggest that these compounds are not responsible for the clastogenicity of MEHP.

We have reported previously (7) that exposure to xanthine and xanthine oxidase causes genetic damage to CHO cells. The effect is prevented by catalase, implying that enzymically generated H<sub>2</sub>O<sub>2</sub> is genotoxic to mammalian cells. This is consistent with the hypothesis (6) that the carcinogenicity of peroxisome proliferators (including DEHP) might be related to the induction of peroxisomal H<sub>2</sub>O<sub>2</sub>-generating enzyme systems in liver cells. To determine whether this mechanism could account for the clastogenicity of MEHP, we employed cytochemical

methods and assays for carnitine acetyltransferase and KCN-insensitive palmitoyl CoA oxidation to investigate peroxisome proliferation in CHO cells treated with MEHP, clofibric acid or BR931. The results suggest that peroxisome proliferation is not induced in these cells and cannot be involved in chromosome damage caused by MEHP. However, we cannot exclude at present the possibility that  $H_2O_2$  generation from intracellular sources other than peroxisomes might be stimulated by MEHP treatment.

Observations on erythrocytes and CHO cells suggest that the cytotoxicity of MEHP may be mediated primarily by attack on cell membranes. Permeability to Trypan Blue, generally used as an indication of cell death, was found to be a reversible phenomenon in CHO cells exposed to MEHP and can probably be regarded as an early indication of membrane disruption. The relationship between MEHP concentration, Trypan Blue permeability, reproductive capacity and chromosome damage (shown in Fig. 1) can be interpreted as demonstrating that chromosome breakage occurs in cells which have suffered considerable but not immediately lethal membrane damage. At concentrations of MEHP above 1.5 mM damaged cells progress to at least one mitotic division but fail to form colonies. Allison and Paton (15) have shown that chromosome damage can be induced in cultured cells by a mechanism involving specific disruption of lysosomal membranes. The observation (16) that exogenous endonucleases can be introduced into normal cells and will induce DNA damage supports the hypothesis that chromosomal breakage can be caused by enzymes released from lysosomes. Although conclusive evidence is lacking, our results are consistent with a lysosomal enzyme-mediated mechanism of clastogenicity of MEHP resulting from its surface active properties. Acid phosphatase, a lysosomal enzyme, was shown to be released by MEHP-treated cells.

The effect of treatment of CHO cells in different culture media may be significant. Although the basis of the effect is unknown, the toxicity of sodium dodecyl sulfate was also modified by the use of different media.

As a mechanism of chemically induced chromosome damage, lysosomal enzyme release may be of little relevance to the effects of MEHP *in vivo* since it is strongly dependent on the composition of the extracellular medium and occurs at relatively high concentrations. Further studies on a range of compounds will be required to determine whether surface-active agents in general have clastogenic potential and whether this potential could be realized *in vivo*. However, even if the chromosomal effects of MEHP are an artifact of cell culture conditions, an

investigation of such effects is clearly of importance for the interpretation of short-term genotoxicity tests.

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