

## Phthalate Ester Toxicity in Human Cell Cultures

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Phthalate Ester Toxicity in Human Cell Cultures, JONES, A. E., KAHN, R. H., GROVES, J. T., AND NAPIER, E. A., JR. (1975) *Toxicol. Appl. Pharmacol* 31, 283-289. Di-2-ethylhexyl phthalate and butyl glycolyl butyl phthalate, plasticizers which can be leached into blood from polyvinyl chloride-containing medical devices, cause significant growth inhibition in cultures of the human diploid cell strain WI-38. The ID<sub>50</sub> (dose which causes 50% growth inhibition in tissue culture) values for di-2-ethylhexyl phthalate and butyl glycolyl butyl phthalate were 70  $\mu\text{M}$  and 12  $\mu\text{M}$ , respectively, for WI-38 cells. Toxic effects were greater in a replicating cell population than in a nonreplicating, confluent cell layer. WI-38 cells which were grown in 160  $\mu\text{M}$  di-2-ethylhexyl phthalate for 3 days, and subsequently subcultured into control medium, showed only 60% of control growth after 5 days in control medium. Cells treated with 14  $\mu\text{M}$  butyl glycolyl butyl phthalate for 3 or 5 days exhibited growth equivalent to the controls when subcultured into control medium. Toxic levels for di-2-ethylhexyl phthalate were within the range of concentrations found in blood which has been stored in polyvinyl chloride blood bags for up to 21 days at 4°C. ID<sub>50</sub> values were reported for several other phthalate esters and for two nonphthalide compounds which are leachable from certain polyvinyl chloride plastic medical devices.

Phthalates, which are used as plasticizers for many polyvinyl chloride (PVC) items in medical and laboratory use, have recently come under close investigation (Autian, 1973). It has been shown that measurable concentrations of di-2-ethylhexyl phthalate (DEHP)<sup>4</sup> accumulate in blood stored in PVC bags for up to 21 days at 4°C (Jaeger and Rubin, 1970a), and in anticoagulant solution stored in PVC bags (Jaeger and Rubin, 1970a, Guess *et al.*, 1967). Also, DEHP has been found in the tissue of deceased patients who had recently received transfusions (Jaeger and Rubin, 1972). Certain formulations of PVC tubing used in hemodialysis units have been shown to release DEHP into circulating plasma (Jaeger and Rubin, 1970b). Other tubing formulations have revealed the presence of another plasticizer, butyl glycolyl butyl phthalate (BGBP) (Jaeger and Rubin, 1970b; Trimble *et al.*, 1966).

Both DEHP and BGBP show low acute toxicity in rats (Singh *et al.*, 1972; Calley *et al.*, 1966) but show some teratogenic effects when administered to rats in large doses

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<sup>4</sup> The more correct nomenclature is bis(2-ethylhexyl) phthalate.

(Singh *et al.*, 1972). However, in order to determine the possible effects of these compounds on human tissues, it is important to study their subacute toxicity by tissue culture assay. We have chosen to use the human diploid cell strain WI-38 (Hayflick, 1965) to study changes in total cell protein and in gross morphology. Both DEHP and BGBP, which were rigorously purified, were added directly to the culture medium similar to unpublished procedures on mouse fibroblasts (Autian, 1973). While previous *in vitro* studies have described an agar-overlay method (Guess *et al.*, 1965; Guess and Haberman, 1968), the validity of this approach with DEHP and BGBP is open to question. These compounds have a very low water solubility, and may not completely permeate the agar layer. One study using this method noted that the more soluble phthalates were most toxic (Calley *et al.*, 1966), while no toxicity was reported for DEHP. Our experiments provide a more direct approach for determining the toxic effects of phthalate esters on human cells.

#### METHODS

Human diploid cell strain WI-38<sup>5</sup> was grown in Eagles basal medium<sup>6</sup> with 10% calf serum<sup>7</sup>. The cultures used for protein determination were grown in Falcon T-25 flasks<sup>8</sup> which were incubated at 37°C and maintained at pH 7.2–7.4. Cell protein was determined by the method of Oyama and Eagle (1956). Cells grown on cover slips were stained with oil red O and hematoxylin.

Since both DEHP<sup>9</sup> and BGBP<sup>10</sup> solubilize more readily in calf serum than in culture medium alone, these phthalates were first stirred into the calf serum moiety of the medium for 22 hr, which was, in turn, stirred into the culture medium for an additional 22 hr. Even by this method, the compounds were incompletely solubilized, and were visible as an emulsion. To determine the true amount in solution, the emulsion was removed by centrifugation at 3000 rpm for 10 min, and the aqueous samples were extracted by the Folch technique (Folch *et al.*, 1957). Quantitation of these samples was performed by gas liquid chromatography (glc) using a 1% SE30 column (1.8 m × 4 mm) on 80 to 100-mesh Chromosorb-W, at 200°C. Assays were also conducted on a computerized glc mass spectrophotometer (Sweeley *et al.*, 1970).

The principal phthalates used in this study were DEHP and BGBP. However, the following additional phthalates (Table 1) were studied to determine ID50 values (dose which causes 50% growth inhibition in tissue culture): di-*n*-butyl phthalate<sup>11</sup>, di-iso-butyl phthalate<sup>12</sup>, dimethoxyethyl phthalate<sup>13</sup>, and di-*n*-octyl phthalate<sup>14</sup>. Two non-phthalide, PVC leachable compounds also tested were di-2-ethylhexyl adipate<sup>15</sup> and

<sup>5</sup> Obtained from L. Hayflick, Stanford, CA.

<sup>6</sup> Gibco, formula G-13, Grand Island, NY.

<sup>7</sup> Flow Laboratories, Rockville, MD.

<sup>8</sup> Falcon Plastics, Oxnard, CA.

<sup>9</sup> Aldrich Chemical Co., Milwaukee, WI.

<sup>10</sup> Matheson, Coleman, and Bell, Norwood, OH.

<sup>11</sup> Sargent-Welch, Skokie, IL.

<sup>12</sup> Aldrich Chemical Co., Milwaukee, WI.

<sup>13</sup> Fluka AG, Chemische Fabrik, Germany.

<sup>14</sup> Eastman Kodak, Rochester, NY.

<sup>15</sup> Synthesized from 2-ethylhexanol (Aldrich) and adipic acid (Eastman Kodak) and purified as above.

2,6-di-tert-butyl cresol<sup>16</sup>. All compounds were chromatographed and rigorously purified using Woelm silica gel<sup>17</sup> with a benzene : ethyl acetate ratio of 95 : 5.

TABLE 1  
ID50 VALUES FOR A SERIES OF PHTHALATE ESTERS AND LEACHABLE NONPHTHALIDE COMPOUNDS  
USING WI-38 CELLS

Compound	Molecular weight	ID50 <sup>a</sup> $\mu\text{M}$	Solubility mol/liter
Phthalate esters			
Di- <i>n</i> -butyl phthalate	278	135	0.008
Di-iso-butyl phthalate	278	85	Very low
Dimethoxyethyl phthalate	282	3500	0.040
Butyl glycolyl butyl phthalate	336	12	Very low
Di- <i>n</i> -octyl phthalate	391	170	Very low
Di-2-ethylhexyl phthalate	391	70	Very low
Nonphthalide Compounds			
Di-2-ethylhexyl adipate	371	32	Very low
2,6-Di-tert-butyl cresol	220	67	Very low

<sup>a</sup> Media concentrations which cause 50% growth inhibition in tissue culture.

## RESULTS

Analysis by glc revealed that concentrations of 0.25 mM, 0.50 mM, and 1.00 mM DEHP when added to the culture medium, resulted in soluble concentrations of approximately 51  $\mu\text{M}$ , 69  $\mu\text{M}$ , and 160  $\mu\text{M}$ , respectively. The presence of emulsions at all three concentrations and the differences in solubility indicated that the systems had not reached final equilibrium. Measurements of soluble concentrations before addition to the cell layer, and after 5 days in culture, demonstrated that there was further solubilization of DEHP during culture, both within the ranges of the figures reported here.

Cultures treated with 160  $\mu\text{M}$  DEHP for 6 days (Fig. 1) showed a decreased cell density when compared to controls. The flasks treated with 51  $\mu\text{M}$  and 69  $\mu\text{M}$  DEHP showed decreased cell protein (Fig. 2) and a longer generation time, although these cells were still viable at the end of 9 days of treatment. The differences in protein observed between concentrations were statistically significant. Cells treated with 160  $\mu\text{M}$  DEHP were no longer viable by day 9. From these data, it was possible to determine an ID50 value of 70  $\mu\text{M}$  for WI-38 cells. The ID50 value was calculated by plotting the percentage of control protein after 9 days of phthalate treatment vs each phthalate concentration. The phthalate concentration at 50% of control protein was recorded from this plot.

Concentrations of 0.25 mM, 0.50 mM, and 1.00 mM BGBP added to the culture medium resulted in soluble concentrations of 7  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 14  $\mu\text{M}$  BGBP, respectively. The appearance of cells treated with 14  $\mu\text{M}$  BGBP for 6 days was very similar to that shown by cells treated with 160  $\mu\text{M}$  DEHP for 6 days (Fig. 1b). The ID50 value for BGBP in WI-38 cells was 12  $\mu\text{M}$ .

<sup>16</sup> Eastman Kodak Co., Rochester, NY.

<sup>17</sup> M. Woelm, Eschwege, Germany, U.S. distributor ICN Pharmaceuticals, Cleveland, OH.

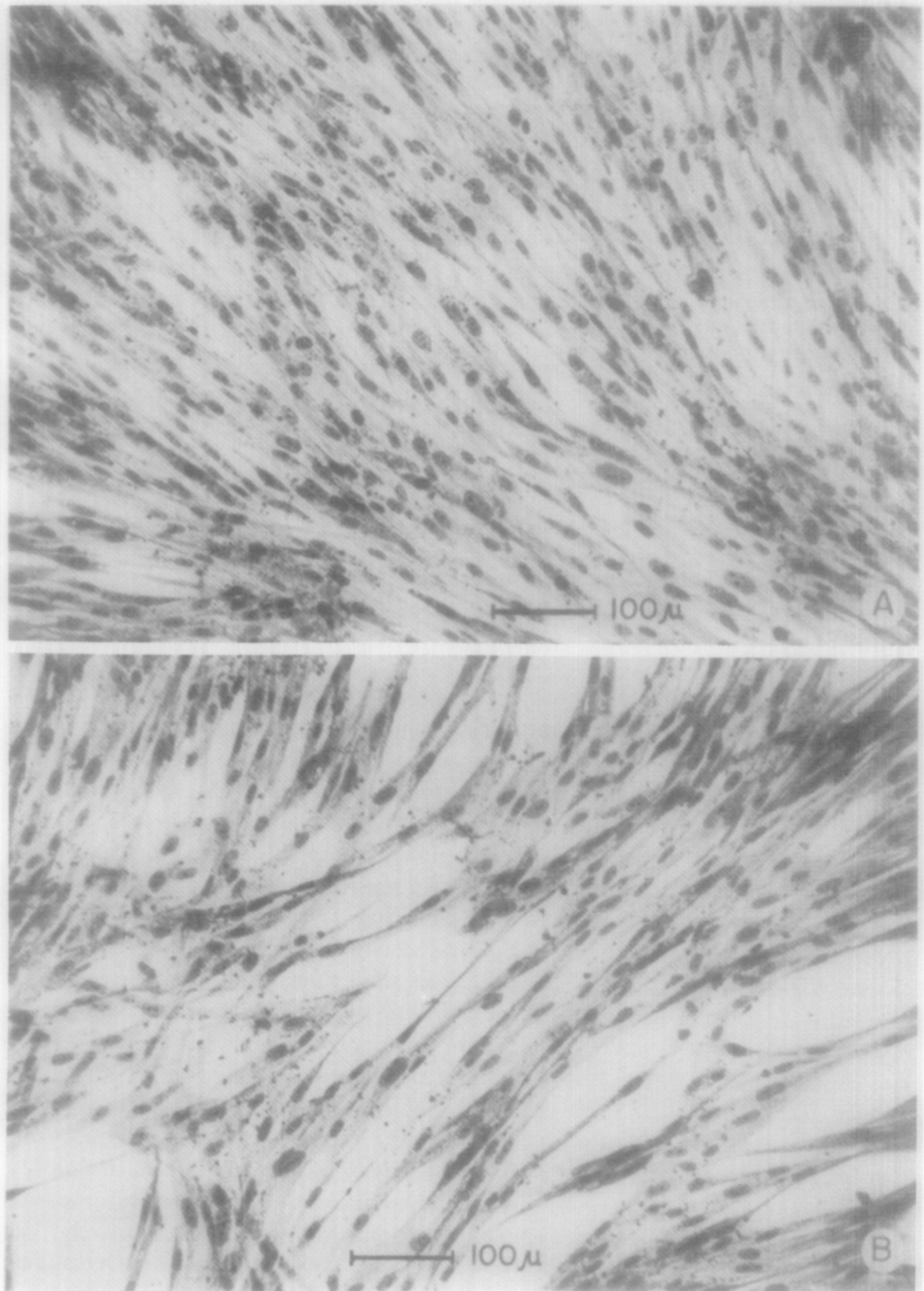


FIG. 1. The effect on monolayer cultures of WI-38 cells of exposure to 160  $\mu\text{M}$  DEHP for 6 days. (A) Untreated control cells. (B) Treated cells. Oil red O, hematoxylin stain.

To test the effects of DEHP and BGBP on high-density cell populations, the cells were allowed to reach an imbricated confluency before the addition of phthalate. At these densities, very little cell division occurred. While these confluent populations were affected by DEHP and BGBP, the effects on cell protein were proportionately less than those exhibited by replicating populations (Fig. 2). Structurally, the cells appeared thinner after several days of treatment, and open spaces appeared in the previously confluent layer. After 9 days of treatment with 69  $\mu\text{M}$  DEHP, confluent

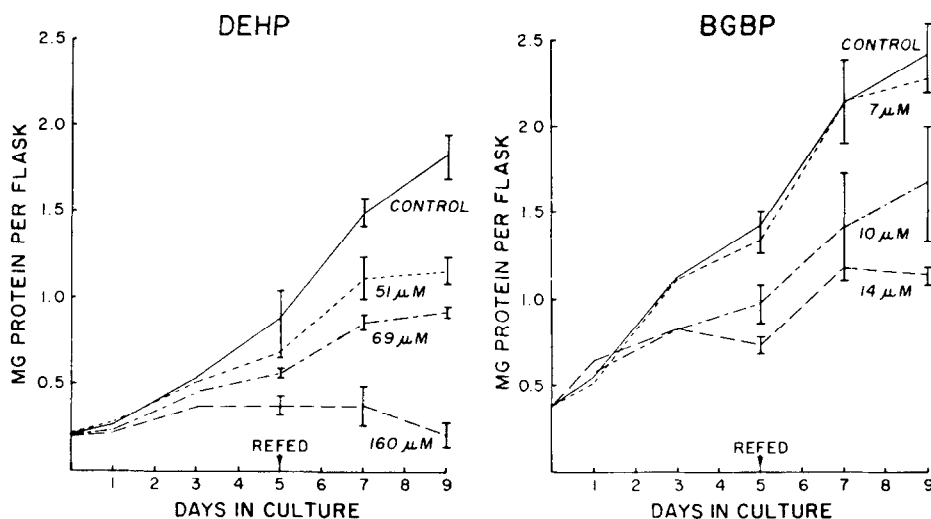


FIG. 2. Protein content of cells treated with BGBP (Right graph) and DEHP (Left graph). Each point represents the mean value for three sample flasks. Brackets indicate  $\pm 1.5$  SE of the mean.

populations exhibited 61% of control protein, whereas replicating populations showed only 50% of the control value. Treatment for 9 days with 14  $\mu\text{M}$  BGBP resulted in 72% of control protein in confluent populations, in contrast to 48% of control protein in replicating populations.

To determine the reversibility of DEHP and BGBP effects on cultured cells, cells grown in the presence of either compound for 3 and 5 days were subsequently subcultured at equal viable cell densities in the control medium. After 5 days in the control medium, the flasks were washed and total cell protein determined. Cells grown for 3 days in 160  $\mu\text{M}$  DEHP showed only 60% of the control growth after subculture in the control medium. Moreover, the cells treated with DEHP did not fully extend their cytoplasmic processes until 2 days after treatment, the control time being less than 6 hr. In contrast, cells treated with BGBP at 14  $\mu\text{M}$  concentrations for 3 and 5 days did not show decreased viability or a change in membrane attachment after subculture.

Table I shows that the ID50 values for other phthalate esters were also in the micro-molar range. Two nonphthalide leachable compounds which we recently detected in human plasma perfused through hemodialyses units, 2,6-di-tert-butyl cresol, and di-2-ethylhexyl adipate (Jaeger and Rubin, 1973a), were also toxic at low levels.

## DISCUSSION

Our calculated ID50 value of 70  $\mu\text{M}$  for DEHP in WI-38 cells is very similar to the value of 50  $\mu\text{M}$  reported by Autian (1973) for mouse L-929 cells. In addition, our observation that non-replicating WI-38 populations are less affected than replicating ones supports those of Dillingham and Autian (1973). They found that nonreplicating L-929 cells treated with dimethoxyethyl phthalate are more protein stable than replicating ones similarly treated. This may be linked to the findings by Warren and Glick (1968) that there is a protein turnover rate in both dividing and nondividing cells which is independent of mitosis. Dividing cells have an additional protein turnover during mitosis which creates membrane instability and a higher susceptibility to toxicants at this stage.

The water solubility of all but di-*n*-butyl phthalate and dimethoxyethyl phthalate is very low. This fact may account for the low acute toxicity of most of these compounds in whole animals, but does not preclude the demonstrable toxicity evident in a tissue culture system.

Some of the major concerns which have arisen over the use of DEHP, in particular, are the findings that DEHP does not appear to be easily metabolized. Detectable amounts have been found in human tissue (Jaeger and Rubin, 1972), fish tissue (Mayer *et al.*, 1971), and in bovine heart tissue (Nazir *et al.*, 1971). Studies of the absorption and metabolism of related compounds, 2-alkylalkanoates, in rats (Saladin and Napier, 1967), suggest that steric hindrance of the 2-ethyl group in DEHP could alter the absorption and hydrolysis rates of this compound. Thus, it is not unreasonable to expect that some DEHP could be deposited in human tissues.

In blood, the concentrations of DEHP has been reported to reach concentrations of approximately 134  $\mu\text{M}$  after storage for 21 days at 4°C in PVC bags (Jaeger and Rubin, 1972). Equally important, 486  $\mu\text{M}$  DEHP has been detected in platelet concentrates stored at 22°C for only 2 days (Jaeger and Rubin, 1973b). These concentrations are comparable to those found in this study which inhibit WI-38 cell growth completely, causing cell degeneration and death. Our studies of BGBP and other phthalate esters, well as two nonphthalide compounds found leachable from medical devices, indicate a similar toxic effect. Although there have been no observations of clinical problems attributable to the use of these compounds, there is experimental evidence of subtle toxicity due to many phthalate esters found in PVC-containing plastics, as well as the possibility of tissue accumulation of DEHP.

## ACKNOWLEDGMENTS

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