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Effects of Bis(2-ethylhexyl) Phthalate on Chromosomes of Human Leukocytes and Human Fetal Lung Cells

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Abstract ☐ Blood from two male and two female donors was exposed at 37° for 4 hr to concentrations of 60.0, 6.0, 0.6, and 0.06 μg of a widely used plasticizer, bis(2-ethylhexyl) phthalate, per milliliter of blood. The bis(2-ethylhexyl) phthalate was solubilized with polysorbate 80. Appropriate polysorbate and nonpolysorbate controls also were established. Following the 4 hr of incubation, phytohemagglutinin was added and tissue cultures were established. In addition, human fetal lung cells were exposed in tissue culture to a medium containing 6.0 μg/ml of bis(2-ethylhexyl) phthalate in polysorbate 80 for 5 days. Similar controls also were established for these experiments. Analysis of chromosome preparations from all cultures obtained failed to show any increased evidence of isochromatid and chromatid breaks or gaps or abnormal forms at any studied concentration when compared to the control cultures. In addition, analysis of fetal lung cell preparations for aneuploidy failed to reveal any differences between cells from study and control cultures. This study involved a short-term exposure to bis(2-ethylhexyl) phthalate in various concentrations which did not cause damage in leukocytes or fetal lung cells.

Keyphrases ☐ Bis(2-ethylhexyl) phthalate—effect on chromosomes of human leukocytes and fetal lung cells ☐ Phthalate esters—bis(2-ethylhexyl) phthalate, effect on chromosomes of human leukocytes and fetal lung cells ☐ Chromosomes, human leukocyte and fetal lung cell—effect of incubation with bis(2-ethylhexyl) phthalate ☐ Toxicity, potential—bis(2-ethylhexyl) phthalate, effect on chromosomes of human leukocytes and fetal lung cells ☐ Plasticizers—bis(2-ethylhexyl) phthalate, effect on chromosomes of human leukocytes and fetal lung cells

Bis(2-ethylhexyl) phthalate (I) is a plasticizer used in the fabrication of polyvinyl chloride medical devices such as blood storage bags, blood administration assemblies, hemodialysis units, and cardiopulmonary bypass units, and it is one of the most widely used of the phthalate esters. In many instances, it represents 40% or more of the total weight of the finished plastic (1). Roll *et al.* (2) demonstrated by GLC techniques that
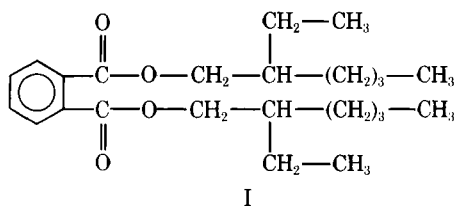
phthalate esters are found almost universally as contaminants on or in items such as laboratory glassware, laboratory chemicals, metal foils, rubber stoppers, and distilled water.

Trimble *et al.* (3) and Guess *et al.* (4, 5) were among the first to note that I leaches out of polyvinyl chloride in the presence of blood or certain other solvents. Since that time, several investigators quantitated the rate of leaching of I from various polyvinyl chloride devices under various conditions (6–10).

Jaeger and Rubin (11) first demonstrated the presence of I in various human tissues following transfusion of blood stored in polyvinyl chloride blood bags. Much research has since been performed to quantify the toxicogenic implications of phthlates in various organs and tissues. Petersen *et al.* (12) showed that I can be detected in liver, lung, and spleen of calves following exposure to cardiopulmonary procedures and in the same organs of humans exposed to prolonged renal hemodialysis.

Recent publications and reviews confirmed that I probably has a low order of acute toxicity (13–15). However, the long-range, subtle toxicogenic potential of this agent is becoming of paramount concern. Guess and coworkers (5, 16) were among the first to suggest potential hazards from long-term exposure of this agent. Observations of cells in tissue culture (19, 20), teratogenic effects (16, 17, 21–24), and effects on reproduction (12, 25) and related studies appear to substantiate this suggestion. While the data are not conclusive and there is incomplete agreement between laboratories, evidence suggests that I and/or its metabolites are toxic at the cellular level, that I interferes with normal reproductive patterns, and that lower molecular weight phthalate esters are teratogenic.

To evaluate possible mechanisms of toxicity and add a dimension to previous studies, human leukocytes and human fetal lung cells were exposed to I at various concentrations and their chromosomes were analyzed for possible changes.

I

Table I—Chromosome Breaks, Gaps, and Abnormal Forms Seen in Leukocytes from Various Cultures for Each Subject[a]

Subject	Concentration of I, µg/ml	Number of Breaks	Percent	Number of Gaps	Percent	Number of Abnormal Forms	Percent
1 (Male)	60.0	2	2.0	5	5.00	0	0
	6.0	1	1.0	7	7.00	0	0
	0.6	2	2.0	4	4.00	0	0
	0.06	1	1.0	0	0	0	0
	Polysorbate control	0	0	0	0	0	0
	Pure control	1	1.0	3	3.00	0	0
2 (Male)	60.0	3	3.0	1	1.0	0	0
	6.0	0	0	2	2.0	0	0
	0.6	2	2.0	1	1.0	0	0
	0.06	0	1.0	2	2.0	0	0
	Polysorbate control	0	0	4	4.0	0	0
	Pure control	0	0	3	3.0	0	0
3 (Female)	60.0	4	4.0	3	3.0	1	1.0[b]
	6.0	3	3.0	6	6.0	0	0
	0.6	0	0	0	0	0	0
	0.06	1	1.0	1	1.0	0	0
	Polysorbate control	1	1.0	1	1.0	0	0
	Pure control	0	1.0	1	1.0	0	0
4 (Female)	60.0	3	3.0	1	1.0	1	1.0[c]
	6.0	1	1.0	2	2.0	0	0
	0.6	2	2.0	3	3.0	0	0
	0.06	2	2.0	3	3.0	0	0
	Polysorbate control	4	4.0	5	5.0	0	0
	Pure control	1	1.0	2	2.0	0	0

[a] Number of cells scored was 100 in each case. [b] Triradial. [c] Ring.

EXPERIMENTAL

For all experiments, I[1] was solubilized in polysorbate 80[2] (1:3) prior to dispersing in fetal calf serum by sonication. Following addition of I to fetal calf serum, the dispersion was filtered through a 0.22-μm filter[3] and diluted such that the concentrations of I desired for a particular experiment would be produced when it was added in 0.1-ml aliquots to 9.9 ml of blood or in 0.2-ml aliquots to 19.8 ml of cells in a tissue culture medium.

Leukocyte Cultures—Sixty milliliters of heparinized blood was obtained from each of two male and two female donors. The donors had been exposed to no medications or known chromosome-damaging agents for at least 1 year and had been exposed to no ionizing radiation for at least 6 months. At the time of obtaining the blood, these individuals were in their early twenties, were in good health, and showed no evidence or stigmata of viral infections.

Each 60-ml sample was divided into six 9.9-ml aliquots. The appropriate I–polysorbate solution was added in 0.1-ml quantities to the first four samples to give final concentrations of 60.0, 6.0, 0.6, and 0.06 μg of I/ml of blood. To the fifth tube, 0.1 ml of polysorbate 80 solution was added to serve as a polysorbate control. Nothing was added to the sixth tube of blood, which served as a pure control tube.

All tubes were incubated at 37° for 4 hr. Phytohemagglutinin then was added, and each tube was placed in cracked ice for 30 min and then centrifuged at 500 rpm for 5 min. The serum containing the leukocytes from each tube was divided equally into three tissue culture flasks, to which Minimal Eagle's medium was added to make a final total volume of 10 ml. All flasks were then incubated at 37° for 72 hr. Two hours prior to harvesting, 0.3 ml of demecolcine[4] was added to each flask. Following this step, harvesting and production of air-dried preparations were carried out.

Slides prepared from each culture were then coded for blind scoring to eliminate the possibility of scorer bias. Each slide was stained with Giemsa stain and permanently coverslipped. Fifty consecutive, intact-appearing metaphase spreads were scored by two observers for total chromosome count, evidence of chromatid and isochromatid breaks and gaps, and the presence of abnormal forms such as tetraploid chromosome count, ring chromosome, and tri- and quadriradials. Each observer dealt with cells from at least two different slides for scoring purposes; thus, 100 metaphase spreads were evaluated for each concentration of I and each control tube.

Fetal Lung Cell Cultures—A stock culture of fetal lung cells was established from a normal appearing 16-week fetus delivered by hysterotomy. For this experiment, an early subculture of cells was used. T-60 flasks were inoculated with 5×10^5 cells, each in Minimal Eagle's medium plus 20% fetal calf serum (heat inactivated). Twenty-four hours after inoculation, all cultures were renewed with medium as follows: two flasks with 19.8 ml of Minimal Eagle's medium with 20% fetal calf serum and 0.2 ml of I–polysorbate 80 solution, and two flasks with 20 ml of Minimal Eagle's medium and 20% fetal calf serum. All flasks were cultured in a 5% CO_2 in air environment at 37°.

Five days after the first renewal, all flasks were renewed with 10 ml of Minimal Eagle's medium with 20% fetal calf serum and 0.8 ml of demecolcine[4]. Eight hours later, harvesting was carried out by removing the monolayer from the glass using 0.25% trypsin in Hank's salt solution. Slides were then prepared by an air-dried method, coded for blind scoring, and scored in the fashion already described.

RESULTS

Table I summarizes the chromosome breaks, gaps, and abnormal forms seen in cells from various leukocyte cultures of the four donor subjects. Since isochromatid and chromatid breaks and gaps were at a minimum, they were combined for statistical purposes. No statistically significant differences were seen between the various concentrations in the two control flasks for each subject. One female subject did show a 4% breakage rate in the 60.0-μg/ml concentration, a 3% breakage rate in at the 6.0-μg/ml concentration, and one triradial in her 60.0-μg/ml concentration culture. The second female subject showed a 3% breakage rate at 60.0 μg/ml but also a 4% breakage rate in the polysorbate control. This subject had a ring chromosome present in the 60.0-μg/ml concentration. However, when all data from each of the four subjects were combined (Table II), no statistically significant differences were noted at the various concentrations when compared to the control cultures.

Table III summarizes the chromosome breaks, gaps, and abnormal forms, as well as percentages of cells with aneuploid counts, seen in the fetal lung cell cultures. Again, isochromatid and chromatid breaks were combined because of the small numbers in each group. No statistically significant differences were noted in any of these categories. Abnormal cells present in each case were tetraploid cells.

DISCUSSION

Since it has been noted that I migrates into blood stored in plastic bags (6, 7) and that it tends to concentrate in certain tissues such as

Table II—Combined Data from All Four Subjects[a] (Leukocyte Experiments)

Concentration of I, μg/ml	Number of Breaks	Percent	Number of Gaps	Percent	Number of Abnormal Forms	Percent
60.0	12	3.00	10	2.50	2	0.5
6.0	5	1.25	17	4.25	0	0
0.6	6	1.50	8	2.25	0	0
0.06	5	1.25	6	1.50	0	0
Polysorbate control	5	1.25	10	2.50	0	0
Pure control	3	0.75	9	2.25	0	0

[a] Number of cells scored was 400 in each case.

Table III—Cells with Breaks, Gaps, and Abnormal Forms Seen in Fetal Lung Cell Experiments[a]

Sample	Number of Breaks	Percent	Number of Gaps	Percent	Number of Tetraploid Cells	Percent	Number of Aneuploid Cells	Percent
I (6.0 μg/ml)	7	3.5	5	2.5	6	3.0	17	8.5
Polysorbate control	9	4.5	5	2.5	3	1.5	11	5.5
Pure control	11	5.5	1	0.5	5	2.5	20	10.0

[a] Number of cells scored was 200 in each case.

the lung (12), it was decided to study the potential chromosome-damaging effect of this agent on both blood leukocytes and fetal lung cells. The 60.0-μg/ml concentration was chosen because it represented the highest concentration used in other experiments wherein I was dispersed in serum by sonication and, following the steps of the experimental protocol, represented a 10-fold dilution of this concentration. Recent data by Turner et al. (26) demonstrated that these levels are within the range of those seen in blood stored in plastic bags. These investigators recently studied the effects of I on chromosomes of leukocytes found in blood-bank blood stored in plastic bags and failed to find any significant chromosome damage.

The 6.0-μg/ml concentration was chosen for the fetal lung experiment because a good mitotic index was desired, but a relatively high concentration seemed appropriate for the 5-day exposure. The leukocyte experiments demonstrated a good mitotic rate at this concentration, whereas the mitotic rate was poor at 60.0 μg/ml.

Polysorbate 80 was chosen as a solvent for I since it is one of the few relatively innocuous agents that could serve this purpose for this agent. Although not known to be cytotoxic, polysorbate 80 was added to a control culture as an added precaution.

Four-hour incubations were used for the blood leukocyte experiments because longer periods had resulted in significant red cell hemolysis in pilot studies designed to expose leukocytes prior to phytohemagglutinin addition to mimic in vivo exposure. Once phytohemagglutinin is added, the mitotic process is initiated and, by the procedure followed, the concentration of I is reduced. However, by this point, the cells have been exposed and some intracellular binding can be anticipated. While exposure to I would vary in vivo depending on the type of medical procedure, the design of the experiment allowed for a variety of concentrations of I, which do occur in many of these circumstances for a specific period.

The fetal lung experiment allowed for a more chronic exposure to I during all stages of the cell cycle.

Chromosome damage and induction of abnormal chromosome forms and aneuploidy are signs of cytotoxicity. It is comforting to note that cells exposed for short periods to I do not show such signs. However, data suggesting toxic effects on cells in tissue culture, teratogenicity, and adverse effects on the outcome of reproductivity, as previously cited, seem to imply that these agents have potentially harmful effects. The results of these studies and those of Turner et al. (26) indicate that these effects are not mediated through a mechanism that damages chromosomes.

Although chromosome damage is often interpreted as evidence for potential mutagenicity, either may occur separately. This report in no way rules out a mutagenic potential of this agent; studies using bacterial and animal systems will be necessary to establish such a possibility. Likewise, this report cannot be interpreted as ruling out a teratogenic effect of the agent since that was outside the scope of the experimental design and specific relationships between chro-

mosome damage and teratogenicity have not been defined. Further animal studies will be necessary to investigate such a potential problem.

Nonetheless, the results of the cited studies imply that exposure to I during pregnancy should be kept to a minimum. Perhaps other substances should be used in the production of blood bags, intravenous tubings, and dialysis equipment when pregnant or potentially pregnant women are to be treated until a clearer understanding of the mechanism of action of I is obtained. It also seems prudent to seek data that describe the specific effects on various tissues after prolonged exposure to these agents.

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Biotransformation of Drugs: Quantitative Structure–Activity Relationships for Barbiturates, Tertiary Amines, and Substituted Imidazoles

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Abstract □ When using free energy-related physicochemical parameters, stimulation of NADPH oxidation by barbiturates and the N-oxidation of tertiary amines was found to be primarily dependent upon the lipophilic character of the substrates as measured by log P, where P is the partition coefficient from either 1-octanol–water or corn oil–water solvent systems. In contrast, the inhibition of epoxidation of aldrin by a series of substituted imidazoles appears to be much more dependent on electronic (σ) and steric (E_s) effects of the inhibitors.

Keyphrases □ Biotransformation, drug—quantitative structure–activity relationships for barbiturates, tertiary amines, and substituted imidazoles, physicochemical properties □ Structure–activity relationships—barbiturates, tertiary amines, and substituted imidazoles, biotransformation, physicochemical properties □ Barbiturates—effect on NADPH oxidation, quantitative structure–activity relationships, physicochemical properties □ Amines, tertiary—N-oxidation, quantitative structure–activity relationships, physicochemical properties □ Imidazoles, substituted—effect on epoxidation of aldrin, quantitative structure–activity relationships, physicochemical properties □ Metabolism, drug—quantitative structure–activity relationships for barbiturates, tertiary amines, and substituted imidazoles

Since early work (1, 2), the importance of lipophilic character in drug metabolism has been well established and documented (3–6). While there have been many elegant and well-designed *in vivo* and *in vitro* metabolism studies on single drugs, there have been relatively few good investigations of series of compounds to account for their differences in biotransformation in terms of their physicochemical properties. From the tremendous amount of published work on drug metabolism, there are relatively few good sets of data suitable for multiple regression analysis using free energy-related substituent constants. This paper reports the quantitative correlations obtained for the stimulation of NADPH oxidation by barbiturates, the N-oxidation of tertiary amines including chlorpromazine and morphine, and the inhibition of epoxidation of aldrin by substituted imidazoles.

EXPERIMENTAL

The microsomal oxidation data in Table I were obtained from the literature (7–9). The biological data were converted to log C or log 1/C, where C is the molar concentration required to elicit a standard biological response, *i.e.*, log (oxidation rate) in moles per minute per milligram or log $1/I_{50}$. The physicochemical constants used in the regression analysis (Table I) were obtained from the literature or calculated taking advantage of their additive and constitutive nature. All equations assembled in Table II were derived *via* the method of nonweighted least-squares fit using a computer program executed through a computer communicating terminal[1].

RESULTS AND DISCUSSION

Jannson *et al.* (7) studied the effects of a series of substituted barbituric acids on the rat hepatic microsomal monooxygenase system. They reported that there were no significant correlations of the microsomal oxidation with various measured physicochemical properties of the barbiturates such as the oil–water partition coefficient ($P_{o/w}$), pKa, cytochrome P_{450} dissociation constant (K_s), and maximal type I spectral change (ΔE). In contrast, they reported the importance of the partition coefficient in the inhibition of membrane-bound mitochondrial NADH oxidase. They suggested that although the lipid solubility was required for a substance to reach the microsomal cytochrome P_{450}, other properties of the barbiturates were important for determining the affinity of their interaction with the cytochrome system whereas the inhibition of the mitochondrial respiratory chain by uncoupling oxidative phosphorylation could be explained simply in terms of lipoid solubility.

In reanalyzing their results by using the free energy-related log P term instead of the partition coefficient itself, there was an excellent linear correlation (Eq. 2) with the 50% inhibition of NADH oxidation by the barbiturates. The coefficient or the slope associated with the log P term (0.88) is comparable to a similar dependence obtained for identical inhibition by barbiturates (10). The addition of either the pKa or the (log P)² term did not result in a statistically significant improvement in correlation.

From the same work, only slightly improved correlations were found of the stimulation of microsomal NADPH oxidation by barbiturates with their free energy-related parameters (Eqs. 3–6). Closer inspection of the results of the regression analysis revealed that all allyl-substi-

[1] IBM 2741.