Teratogenicity, Mutagenicity, and Cellular Toxicity of Phthalate Esters

by E. O. Dillingham* and J. Autian*

Growing concern that phthalic acid esters (PAE) may present a health hazard and possible deleterious effects on the ecological system has led to increased interest in the more subtle toxicity of those compounds. Although the acute toxicity of PAEs as a group is quite low, the intrinsic cellular toxicity increases with molecular weight. Whether the intrinsic toxicity is great enough to exert a significant effect *in vivo* at the typically low levels of solubility of PAEs is of primary importance to the assessment of their potential hazard. This paper reviews work reported on the teratogenicity of PAEs and new work on the mutagenicity, cellular toxicity, and absorption, excretion, and distribution of those compounds. The relationship of *in vitro* and *in vivo* response is discussed.

Teratogenicity

Teratogenicity of PAEs was examined by Singh, Lawrence, and Autian *(1)*. Six compounds (dimethyl, dimethoxyethyl, diethyl, dibutyl, diisobutyl, and butyl carbobutoxymethyl phthalate) were administered intraperitoneally to pregnant rats in doses of one-tenth, one-fifth, and one-third of the acute LD_{50} dose on the 5th, 10th and 15th days of gestation. Dioctyl and di-2-ethylhexyl phthalate had LD_{50} values of greater than 50 ml/kg and were each administered at dose levels of 5 and 10 ml/kg. The rats were sacrificed on the 20th day of gestation, 1 day prior to expected parturation. The uterine horns and ovaries were surgically exposed to permit counting and recording of the numbers of corpora lutea, resorption sites, and viable dead fetuses. Fetal weights were recorded, and the fetuses were examined for gross malformations. Thirty to fifty per cent of the fetuses not showing gross malformations were selected at random and examined for skeletal malformations. The regimen of PAE administration did not interfere with fertility, as reflected by the ratio of corpora lutea to implantation sites, but there were significant effects upon embryonic and/or fetal development. Embryo-fetal toxicity (dead fetuses plus resorption sites) ranged from 0% for the middle dose of diethyl phthalate to 98.2% for the high dose of dimethoxyethyl phthalate. Fetal malformations ranged from 0% to 100%, both for gross and skeletal abnormalities, with skeletal defects generally being more common. Fetuses from all treated groups were significantly smaller than fetuses of the control group. Absence of tail, anophthalmia, and twisted hind legs were most common gross abnormalities, while elongated and fused ribs and abnormal skull bones were the most common skeletal deformities. The most embryotoxic agent was dimethoxyethyl phthalate, which was the most water-soluble of the esters studied and the compound

*Materials Science Toxicology Laboratories, University of Tennessee Medical Units, Memphis, Tennessee 38103.

showing the lowest intrinsic cellular toxicity in tissue culture (discussed below). Dioctyl and di-2-ethylhexyl phthalates tended to have the least overall adverse effect on embryo-fetal development and were the least soluble of the compounds studied. All of the PAEs studied showed a deleterious effect upon the developing embryo and/or fetus. At one or more of the dose levels employed, some or all of the following effects were observed for each compound: resorptions, gross abnormalities, skeletal malformations, fetal death, or decreased fetal size. The untreated control group showed no resorptions, gross or skeletal abnormalities or fetal deaths. Responses were generally dose-related. Dimethoxyethyl phthalate was distinctly more teratogenic than the other PAEs studied.

In the above cited study, the percentage of skeletal abnormalities was generally correlated with the water solubilities of the compounds. The percentages of gross abnormalities, however, were highest for the most and the least water-soluble compounds, dimethoxyethyl phthalate and di-2-ethylhexyl phthalate, respectively. The percentage of resorptions was highest for the most soluble compound, dimethoxyethyl phthalate, but showed relatively constant percentage resorption (24.1–32.1%) for the other compounds, diethyl and dioctyl phthalate falling below that range. These results suggest that induction of skeletal abnormalities may be dependent on nonspecific effects on bone formation related to the absolute concentrations of the compounds interacting with formative bone interfaces, while gross abnormalities and resorptions may reflect more specific interactions related to both concentrations and the intrinsic cellular toxicity of the compounds. Dillingham, Chambers, and Autian *(2)* have reported that the product of intrinsic toxicity in tissue culture (slope of the dose-response curve) and the molar aqueous solubility shows a constant ratio to the acute LD_{50} in mice for a series of five PAEs. The relative constancy of the percent resorptions in the teratogenic study cited above *(1)* may be related to the fact that intrinsic cellular toxicity of PAEs increases proportionally as water solubility decreases. The bimodal distribution of gross abnormalities probably reflects the specific nature and relative ease of detection of gross deformity. A degree of specificity or a disproportionate increase in intrinsic toxicity with decreasing solubility is suggested.

Mutagenicity

In view of the teratogenic potential of PAEs the possible mutagenicity of those compounds was investigated by Singh, Lawrence, and Autian by dominant lethal assay in mice *(3)*. Their unpublished work is summarized here.

Mutagenic effects of di-2-ethylhexyl phthalate (DEHP) and dimethoxyethyl phthalate (DMEP) were investigated by intraperitoneal administration of a single dose of the respective undiluted esters to male (ICR) mice immediately prior to the initiation of a mating period in which 10 treated males were mated (two females per male) each week for 12 weeks. The results of that study are summarized in Table 1. The dose levels employed were one-third, one-half, and two-thirds of the acute LD_{50} dose. A parallel untreated mating group was maintained as a control (240 matings). Shortly before expected parturation, the pregnant females were sacrificed and the uterine horns exposed surgically to permit recording of the numbers of corpora lutea, total numbers of implantations, preimplantation losses (by difference between those two parameters), resorption sites (early fetal deaths), dead fetuses (late fetal deaths), and viable fetuses. Since there was a significant antifertility effect with both compounds, only total (early plus late) fetal deaths were employed in the statistical test for dominant lethality. The results reported are, therefore, a conservative estimate of dominant lethal effects since some of the preimplantation losses may have been due to dominant lethal mutation. The significant reductions in mean live fetuses per pregnancy and mean implants per

Table 1. Antifertility and mutagenic effects of phthalate esters.

Phthalate ester	Dose, ml/kg	Pregnancies, % of control	Implants[a]	Fetal deaths[a]	Live fetuses[a]
None	Untreated control	71	11.4	0.45	11.0
DEHP	12.78	56	11.3	0.80[b]	10.5
	19.17	59	11.1	0.65[c]	10.5
	25.56	22	10.5[a]	0.79[b]	9.6[b]
DMEP	1.19	70	11.1	0.35	10.8
	1.79	75	11.2	0.56	10.7
	2.38	35	10.6[b]	0.74[b]	9.8[b]

[a] Per pregnancy, mean values.

[b] Greater than 99% confidence (Student *t* test).

[c] Greater than 95% confidence (Student *t* test).

pregnancy at the high dose levels of both compounds are consistent with the finding of a significant level dominant lethal mutation for both compounds. Mean implants per pregnancy and mutagenic effects tended to be bimodally distributed in the DEHP experiment, significant differences from the control showing up during weeks 1–5 and 10–12. A similar distribution was observed for mean implants per pregnancy in the DMEP experiment; however, the mutagenic effect was observed only at the high dose level and distributed in the period between weeks 2 and 9. The mutagenic effect of DEHP was significantly greater than for DMEP in contrast to the teratogenic effects of those compounds.

Tissue Distribution, Dermal Absorption and Excretion of PAE

Urinary excretion of ^{14}C-DEHP by mice receiving undiluted ester intraperitoneally (IP) and saturated saline solution of the ester intravenously (IV) was studied by Dillingham and Pesh-Iman and reviewed by Autian *(2)*. The observed cumulative excretion of the compound for the two experiments is given in Figure 1. The IP dose was 5 ml/kg; the IV dose was lower by a factor of 10^4. There was a very slight peak of excretion at 24 hr following IV administration and a slightly higher cumulative excretion compared to the cumulative excretion following IP administration, but the pattern of excretion was closely parallel for the two routes.

A significant diuretic effect was observed following IV administration of the DEHP saturated saline solution and a distinct suppression of urinary excretion following IP administration of the undiluted ester. It was

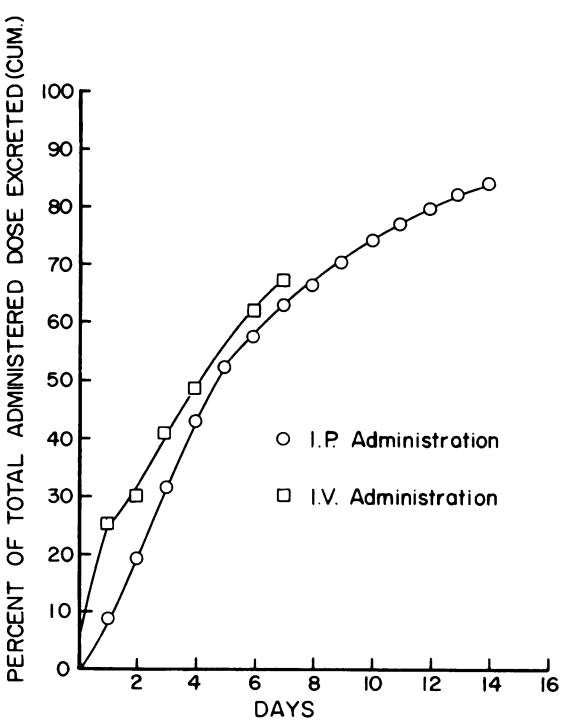

FIGURE 1. Cumulative urinary excretion of DEHP by mice.

observed that the IV administration of DEHP saturated saline to mice which had been pretreated (a single IP dose of undiluted DEHP 3 days prior to IV administration, doses as in the above experiments) did not lead to diuresis. The urinary output remained at approximately the same level as in the IP experiment.

The daily urinary excretion rates for the above three experiments are given in Figure 2. Excretion showed a first-order decline following IV administration to both untreated and IP-pretreated animals. There was a peak of excretion at three days following IP administration. In view of the relatively large dose of DEHP administered IP (approximately 10^3 times the quantity required to saturate the aqueous phase of a 25-g mouse, based on water solubility) and in view of the fact that only 23% of the administered dose was excreted at the end of 3 days, a change in the intrinsic rate of excretion after 3 days was suggested or there may have been a significant shift in the equilibrium between the large PAE pool and the circulating fluid compartment. To examine the question of equilibrium between the circulating compartment and the DEHP pool, the DEHP administered in the above IP–IV experiment was ^{14}C-carboxyl-labeled. The daily excretion rate, (Fig. 2) and the cumulative excretion (Fig. 3) following IV administration of the labeled compound was calculated on the assumption that the IV administered labeled ester reached rapid equilibrium with the unlabeled DEHP pool (approximately 77% of the IP administered dose, based on the data of Figure 1; the excretion of unlabeled compound was assumed to follow the same time course as the labeled compound, and the data of Fig. 1 are used in Fig. 3 for the IP cumulative excretion of unlabeled compound). The close agreement of the IV and IP excretion curves (Fig. 3) is consistent with the assumption of rapid equilibrium and with the conclusion that the DEHP pool at 3 days had not been metabolized to any large degree. Approximately 83% of the total administered dose (IP) was excreted by 14 days, and no significant radioactivity was found in the urine of mice after 35 days. The consistency of the excretion data based on the assumption of free equilibrium between the circulating compartment and the total residual pool suggested that a shift in mechanism of excretion was involved rather than time dependencies of transport. A small amount of the radioactive compound was found in the feces but not enough to account for any significant depletion of the pool. The vapor pressure of the ester was too low to assume any significant losses by exhalation.

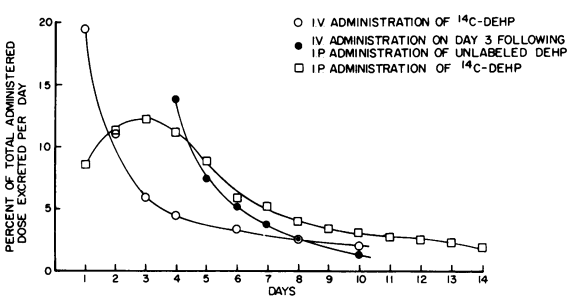

FIGURE 2. Rate of urinary excretion of DEHP by mice.

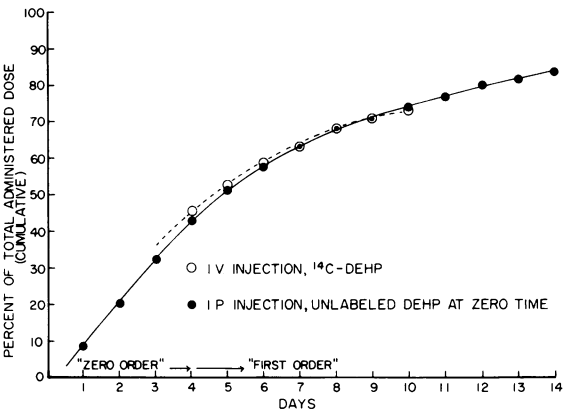

FIGURE 3. Urinary excretion of ^{14}C-DEHP administered IV to mice on day 3 following IP injection of unlabeled DEHP.

Dermal absorption of ^{14}C-DEP (diethyl phthalate, carboxyl-labeled) was determined by application of the ester to the skin of rabbits and assay of radioactivity in the urine daily. Of the total applied radioactivity, 9% was found in the urine at 24 hr, 14% at 48 hr, and 16–20% at 72 hr. A similar experiment was carried out with DEHP, except that radioactivity in organs

was examined by radioautography (discussed below). Detectable radioactivity in several tissues indicated absorption of DEHP through the skin. A quantitative estimate of the amount absorbed was not obtained.

The animals employed in each of the above experiments were sacrificed at the end of the experiment and organs and tissues collected for determination of PAE distribution by assay of radioactivity. At 7 days after IV administration of ^{14}C-DEHP saturated saline (Fig. 1), the highest specific activity (dpm/g tissue) was found in the lungs, lesser but significant amounts being found in the brain, fat, heart, and blood, with no apparent preferential deposition in fatty tissue. Radioactivity observed in the lung was significantly higher (by a factor of 10–20) than could be accounted for by residual blood in the tissue. Fourteen days following IP administration of ^{14}C-DEHP (Fig. 1), lungs showed the highest specific activity relative to other organs and tissues, although the absolute amount of radioactivity was less than at 7 days. The distribution of the total administered IP dose found in organs and blood at 14 days was 0.34% in lung, 0.20% in brain, 0.18% in heart, and 0.54% in blood. At 7 days following IV administration, the distribution of total administered dose in organs and tissues was 1.15% in lungs, 0.11% in brain, 0.13% in fat, 0.09% in heart, and 1.25% in blood. Distribution 7 days following IV administration to IP-pretreated mice (Fig. 3) was very similar.

Organs and tissues were collected from rabbits after three days of dermal exposure to ^{14}C-DEHP. Radioactivity was detected by radioautography of tissue sections of lung, heart, liver, kidney, gonads and spleen. The radioactivity was not random but showed distinct intracellular localization. Skin and subdermal fatty tissue at the site of application showed no radioactivity, suggesting considerable loss of radioactivity from tissues during fixation (formalin). Radioactivity was not uniformly distributed in all cells and, where it was detected, appeared to be localized in the nuclear region.

Cellular Toxicity of Dimethoxyethyl and Dimethyl Phthalate

A study of the cellular toxicity of DMEP and DMP (dimethyl phthalate) was carried out by Wu, Dillingham, and Autian, Materials Science Toxicology Laboratories. That work has been submitted for publication *(4)*. The following is a summary of the principal findings.

The objective of the investigation was to determine the intrinsic susceptability of mammalian cells to the toxic effects of DMEP and DMP in tissue culture. Growth and protein synthesis were followed with respect to cellular toxicity and observed *in vivo* response.

Mouse fibroblast L-cells, NCTC clone 929 strain L (Earl), were employed in the investigation. The cultures were maintained and grown on Eable's medium to which appropriate concentrations of toxicant were added. The per cent growth as compared to the controls was calculated from protein assay, an essentially linear relationship being found between total protein and cell number. Two types of experiments were carried out to determine the sensitivity of replicating and nonreplicating cells. The replicating cell culture was established by inoculation with 2×10^5 cells per assay tube. A parallel experiment involving use of an inoculum of 2×10^6 cells per assay tube resulted in an essentially nonreplicating culture *(5)*. Dose response was determined over 144 hr and 96 hr, respectively, for the two systems. Protein turnover in the replicating system was determined by an experiment in which the cells for inoculum were labeled by growing them in the presence of ^{14}C-L-leucine. The labeled cells were added to unlabeled assay medium, and the decline in specific activity of protein was followed for 96 hr. The difference between the observed specific activity and that expected from dilution by newly synthesized unlabeled protein was used to calculate the per cent turnover. The above experiments were carried out with DMEP. An experiment was also carried out with dimethyl phthalate (DMP) in a replicating cell culture system by employing a ran-

domly labeled amino acid mixture (algal protein hydrolyzate). Label was added at zero time, and the total radioactivity of the cultures was followed in the untreated controls and in cultures treated with 4.185 x 10^4M DMP over a period of 96 hr. One set of DMP-treated cultures (unlabeled medium) was pulse-labeled with ^{14}C-Amino acids for 2 hr at 24-hr intervals.

The dose response obtained in the replicating system (by protein assay) is shown in Figure 4. Fourfold increase in cell numbers was obtained in 72 hrs and fivefold increase by 144 hr. There was a significant dose response over the entire period with a distinct increase in growth inhibition above 0.004*M* DMEP. The onset of significant differential inhibition was between 24 and 48 hr. At the highest concentration of DMEP, growth was completely inhibited until after 72 hr, a decrease in cell numbers (total protein) being apparent at 144 hr.

In contrast to the replicating cell culture, the nonreplicating cultures (Fig. 5) showed essentially no response at the lower concentrations but showed a significant response after 24 hr at 0.008*M* DMEP. The relative insensitivity of replicating cells prior to the first replication (24 hr) is apparent in the experiment carried out with DMP (Fig. 6). The radioactivity data of that experiment closely followed total protein by independent protein assay for the continuous labeling experiment, indicating an initial increase (due to the first relatively uninhibited cell division) followed by a decline in cell number which suggested active lysis of the cell population after 24 hr. The rate of amino acid uptake over the 96-hr period (pulse labeling) was inversely related to the degree of lysis (suppression of total protein). The observed apparent increase in amino acid uptake at 96 hr was probably due to decrease in the unlabeled pool of L-leucine in the

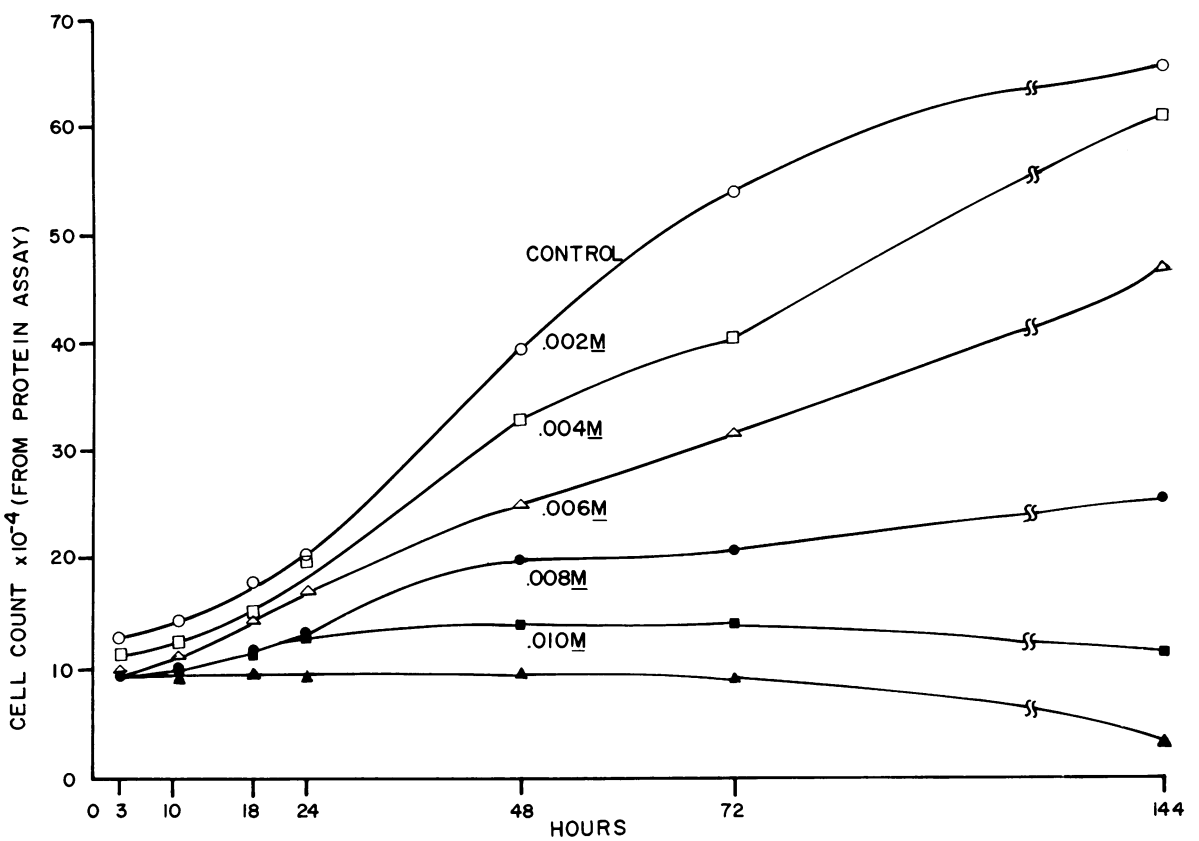

FIGURE 4. Growth inhibition of replicating cells in the presence of DMEP.

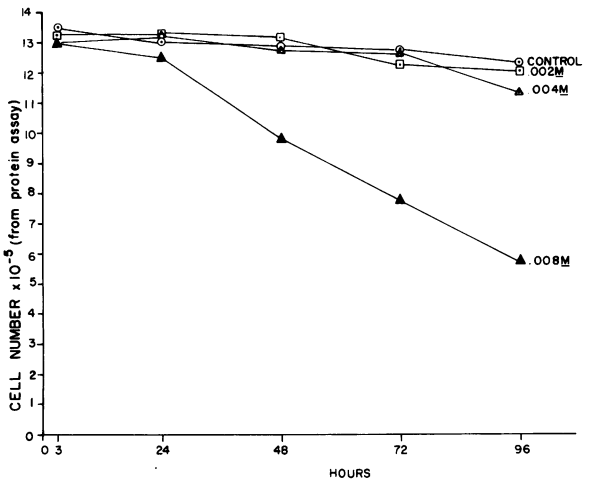

FIGURE 5. Growth inhibition of nonreplicating cells in the presence of DMEP.

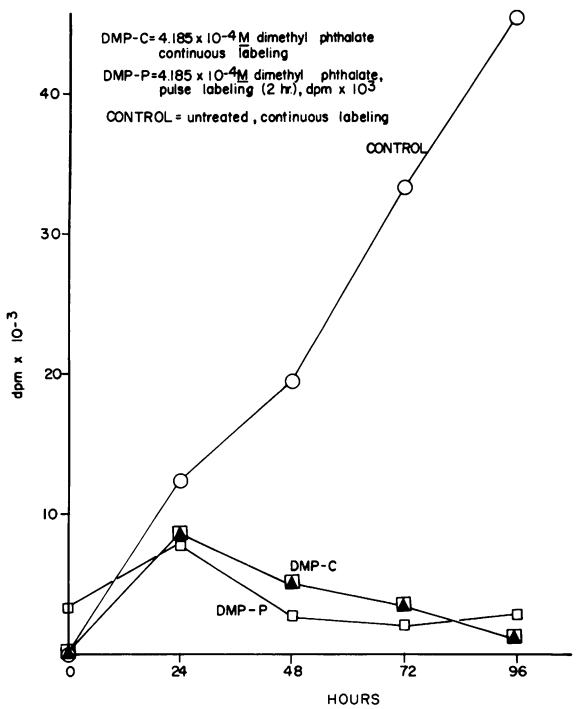

FIGURE 6. Growth inhibition of replicating cells in the presence of DMP.

culture medium at the time of pulse labeling. The true rate of amino acid uptake was increasingly less than the apparent values with respect to time.

Protein turnover in the replicating culture is given in Table 2. The turnover of cell protein was a function of both age and toxicant concentration. The salient feature of the data over the period of 48 to 96 hr was the approximately constant turnover values with decreasing concentration with respect to time, e.g., 0.01*M* at 48 hr gave 31%, 0.008*M* at 72 hr gave 30%, and 0.006*M* at 96 hr gave 29%. Also, the 24-hr turnover values along the same lines of comparison are approximately half those at 48 hr, indicating a significantly lower rate of turnover in the first 24 hr, a period of lower sensitivity to the toxicant. The relative protein turnover with respect to time and concentration is given in Tables 3 and 4, respectively. The significant difference of the control is apparent as well as the significant difference in protein turnover in the first 24 hr as compared to later times. The relative turnover remained approximately constant with respect to time after 48 hr at each concentration. There was, however, a decreasing effect on turnover with respect to concentration. Since the doubling time for the L-cell was approximately 20–24 hr, the degree of suppression of growth appears to

Table 2. Effect of dimethoxyethyl phthalate on protein turnover of L-cells.

DMEP concn, mole/l.	Protein turnover, %			
	24 hr	48 hr	72 hr	96 hr
0.000	1.8	6.2	11	18
0.002	4.7	8.8	15	17
0.004	4.4	14	15	24
0.006	5.5	18	22	29
0.008	7.8	22	30	40
0.010	8.2	31	46	57

Table 3. Relationship of protein turnover at 96 hr to turnover prior to 96 hr in a replicating cell culture treated with DMEP.

DMEP concn, mole/l.	Ratio of protein turnover		
	96 hr/24 hr	96 hr/48 hr	96 hr/72 hr
0.0	10.0	2.9	1.6
0.002	3.6	1.9	1.1
0.004	5.5	1.7	1.6
0.006	5.3	1.6	1.3
0.008	5.1	1.8	1.3
0.010	7.0	1.8	1.2

Table 4. Relationship of protein turnover at high DMEP concentration to turnover at lower concentrations.

Time, hr	Ratio of turnover at indicated concentrations				
	0.01/0	0.01/0.002	0.01/0.004	0.01/0.006	0.01/0.008
24	4.5	1.7	1.9	1.5	1.1
48	5.0	3.5	2.2	1.7	1.4
72	4.2	3.1	3.1	2.1	1.5
96	3.2	3.4	2.4	2.0	1.4

be related to the number of cell divisions and the concentration of the toxicant. This relationship of toxicant effect and cell division is consistent with the findings by radioautography that systemically distributed DEHP is localized in the nuclear region of cells. Relatively few cells in any given tissue section showed radioactivity by radioautography, and this may be related to the relatively low rate of cell division in somatic tissue.

The distinct difference in susceptability of replicating and nonreplicating cells and the relationship of the toxic effect to cell division provides some insight into the apparent low toxicity of phthalate esters *in vivo* and the manifest teratogenic *(1)* and mutagenic activity of those compounds. In the teratogenic study, the administration of phthalate esters at a concentration of 1/3 of LD_{50} dose (the maximum dose administered) was approximately the amount required to saturate the aqueous compartment of the rat, based on the water solubility of the compound and assuming uniform and complete distribution of the toxicant throughout the body. The lowest administered dose in the teratogenic study was approximately equivalent to the highest administered dose in the present *in vitro* study of DMEP. At that dose level in the teratogenic study, 27.6% embryo resorptions, 2.4% gross abnormalities, and 92.9% skeletal abnormalities were reported, with no apparent toxicity reported for the pregnant female rats. The relative insensitivity of nonreplicating cells to DMEP *in vitro* is consistent with a finding of low *in vivo* toxicity, assuming that the tissues of the mature animal sustain a low maintenance rate of cell division. However, embryonic tissues do undergo extreme fluctuations in cell division rates. At periods in development of specific embryonic tissues where surges of cell division is accompanied by increased protein turnover, phthalate esters can produce a significant toxic response, assuming the intrinsic susceptability of the cell to be the same *in vitro* and *in vivo*.

These findings support the conclusion that DMEP is highly toxic to tissues undergoing significant rates of cell division and that the teratogenic activity of DMEP may be the result of heightened cell sensitivity associated with cell replication in embryonic tissues. The low *in vivo* acute toxicity of DMEP and, perhaps, of other phthalate esters, may be related to the relatively low maintenance rate of cell division and metabolism of somatic tissues as compared to embryonic tissues. A similar mechanism of action was suggested for DMP.

Mutagenicity of PAEs may also be related to the phenomenon of heightened susceptability during periods of active cell division. The postmeiotic period is a period of active cell growth and maturation during spermatogenesis and may involve increased susceptability to toxic (mutagenic) effects. Any tissue which undergoes periodic surges of cell division may be particularly liable to the toxic effects of PAEs. It would be informative to investigate the effects of PAEs on tissues (such as spleen, bone marrow, wound tissue, and gonads) of mature animals where cycles of stimulated cell division and metabolic activity are encountered.

REFERENCES

1. Singh, A. R., Lawrence, W. H., and Autian, J. 1972. Teratogenicity of phthalate esters in rats. J. Pharm. Sci. 61: 51.
2. Autian, J. 1972. Toxicity and health threats of phthalate esters: review of the literature. National Library of Medicine, Toxicology Information Program, Oak Ridge National Laboratory, Oak Ridge, Tenn. in press.
3. Epstein, S. S., and Shafner, H. 1968. Chemical mutagens in the human environment. Nature 219: 285.
4. Wu, C. H. Dillingham, E. O., and Autian, J. Toxicity of dimethyl and dimethoxyethyl phthalate in mammalian tissue culture. J. Pharm. Sci. in press.
5. Warren, L., and Glick, M. C. 1968. Membranes of animal cells. II. The metabolism and turnover of the surface membrane. J. Cell Biol. 37: 729.