INTERACTION OF PLASTICIZERS WITH MAMMALIAN CELLS

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Introduction

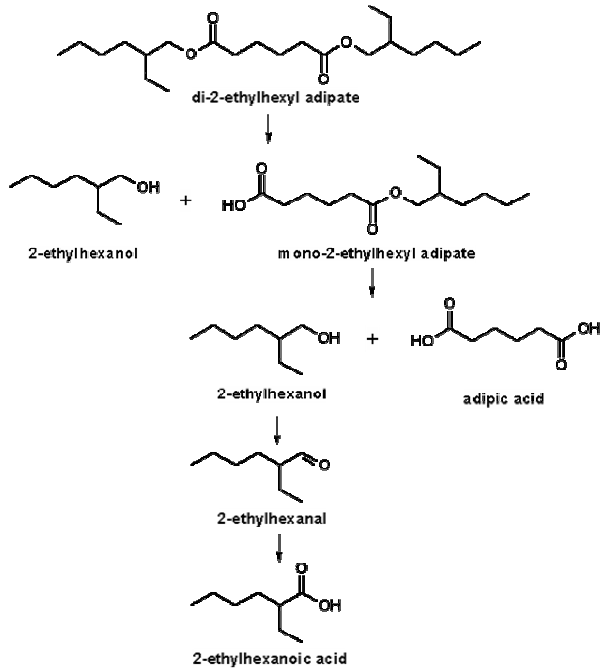
The use of plastic raw material for manufacturing domestic and construction products has been realistic partly due to additives that are incorporated to enhance application specific properties [1]. Plasticizers are one class of such small molecular weight additives that alter the polymer's workability, flexibility and elongation properties [1, 2]. Unfortunately these compounds have been identified as ubiquitous pollutants since they easily leach out their polymer matrices in large amounts.

Several types of plasticizers are currently used in various applications and can make up as much as 40% of common plastics. The most common plasticizer is di(2-ethylhexyl) phthalate (DEHP), used in the production of polymerized vinyl chloride (PVC) plastic [1, 2]. DEHP belongs to the family of phthalate ester plasticizers and accounts for 50% of the total production of all plasticizers [3], or about 500 thousand tons per year [4]. Another family of plasticizers which lack the presence of an aromatic group is the adipate esters. The most prominent member of this family is di(2-ethylhexyl)adipate (DEHA), which is used in PVC films to maintain flexibility over a wide range of temperatures [1].

The major problem encountered with the use of plasticizers is their continuous leaching out of the polymer matrix into other products and the environment, since they are not chemically bonded [2]. This problem is clear especially in landfills case studies, where the plasticizer can leach out into the soil and accumulate at concentrations as high as 100 µg/g of soil [5]. Effluents of the plasticizer industry represent another large source of plasticizers with as much as 1.5 mg/L content [6]. With time these chemicals will diffuse to contaminate most soil and aquatic ecosystems, making plasticizers ubiquitous compounds. Research has shown that plasticizers are generally not good candidates for any sort of biotransformation therefore they will continue to accumulate [7].

Some common soil organisms that naturally degrade hydrocarbons or benzene rings are capable of at least partially degrading these compounds [1]. It was recently reported that partial degradation of the DEHA and DEHP in the presence of a carbon source, hexadecane, is possible, to the monoesters, and 2-ethylhexanol and 2-ethylhexanoic acid [1], which are unfortunately more toxic than the parent compounds (Figure 1).

Figure 1. Pathway for the production of plasticizer metabolites from the degradation of di 2-(ethylhexyl) adipate (DEHA) by bacteria, yeast and fungi [1].



Mammalian metabolism. The first experiments involving mammalian organisms metabolizing plasticizers were done in 1973 by Albro and Fishbein [8] after the discovery of phthalate esters leaching from storage bags into blood [9].

The rat metabolism data at the time suggested that DEHP is first hydrolyzed to MEHP, which is then transformed into other monoester derivatives after undergoing ω-oxidation and (ω-1)-oxidation, postulated to take place in the liver. The researchers also suggest that the monoester is handled by the organism as a fatty acid, because α- and β- oxidation is initially impossible, so ω-oxidation results. No mention is made of the

discovery of the acid or alcohol seen in bacterial degradation, although it is always assumed that 2-ethylhexanol is always produced at the same time DEHA is hydrolyzed.

The metabolism of DEHP and excretion of metabolites in urine has also been studied in many other mammalian species, either intravenously or by ingestion. A large fraction of metabolites are excreted as glucuronide conjugates in humans [10]. Glucuronidation is an example of how a healthy body employs many different detoxification pathways, in the liver and elsewhere, of which glucuronidation by the enzyme uridine 5'-diphosphoglucuronyl transferase (UGT) is one. The product of this reaction is a glucuronic acid conjugate.

The study by Lhuguenot et al. in 1985 [11] investigated the metabolism in rats of DEHP and its monoester MEHP both in vivo and in vitro. Data for in vitro metabolism by rat hepatocytes however was only collected for the monoester and not for the parent compound since it was assumed that DEHP is hydrolyzed in the rodent intestine. It was found that at concentrations of 50 to 500 μM MEHP was metabolized to compounds differing from MEHP in one carbon or hydrogen atom, and that there was a time and concentration dependency. Another study assessed the amount of hydrolysis in the skin while plasticizers similar to DEHP are being absorbed [12]. The authors found that after during exposure of the skin to plasticizers dimethyl phthalate, diethyl phthalate, and di-n-butyl phthalate, the transdermal metabolism of human and rat skins produced the corresponding monoester, as well as some phthalic acid.

Enzymatic studies. Many animal enzymatic studies have been performed to find out where the strongest carboxylesterase enzymes are found, mainly in order to determine where the insoluble phthalate diester is absorbed by the body as the more soluble monoester or the alcohol [13, 14, 15, 16, 17]. Results with rat tissue extracts show that most of the enzyme is present in the pancreas, liver and the intestine [13], suggesting that most of the phthalate diester is hydrolyzed in the gut. Another study by Lake et al. [16] investigates the hydrolysis by hepatic and intestinal preparations from rats, baboons, ferrets and humans (only intestinal). The results show that the hydrolysis of DEHP is performed about 50 times faster by liver enzymes in all test animals. It was also noticed that gut enzyme activity correlates inversely with the alkyl side chain length of the phthalate diester. The liver, kidney, testes, and blood were identified as sites of DEHP metabolism or utilization after 14-day oral exposure of rats to a 2,000 mg/kg/day dose containing 14C-DEHP labeled in the phenyl ring [18].

The activity of human liver enzymes has been investigated by Mentlein and Butte [17]. Carboxylesterases were highly purified from human liver microsomes and mixed with phthalate ester emulsions in Triton-X 100. The obtained solution after incubation was assayed for amount of carboxylic acids.

Toxicology. DEHP is best classified as a non-genotoxic epigenetic chemical that can reversibly inhibit gap junctional intercellular communication and thereby alter homeostatic control of cell proliferation, cell differentiation, and programmed cell death [19]. A characteristic effect of exposure to DEHP in rodents, particularly rats and mice, is an increase in liver weight, associated with both morphological and biochemical changes. Liver enlargement is due to both hepatocyte hyperplasia and hypertrophy. Morphological examination reveals an increase in both the number and the size of peroxisomes in the liver. Peroxisomes are cytoplasmic organelles found in the all kinds of organisms. Peroxisomes contain catalase, which destroys hydrogen peroxide, and a number of fatty-acid oxidizing enzymes, one of which, acyl CoA oxidase, generates hydrogen peroxide [20]. The mechanism by which peroxisomes proliferation induces liver carcinogenicity is still not well understood, but it seems that they induce oxidative stress due to hydrogen peroxide imbalance, or cell proliferation due to a transient increase in replicative DNA synthesis and cell division. Humans are non-responsive to peroxisomal proliferation and are probably less susceptible to liver cancer than rodents due to the species specificity of the mechanism. It is therefore likely that humans will not be at such high risk of liver cancer due to DEHP exposure.

DEHP also induces testicular toxicity characterized by structural as well as biochemical alterations in the testis. Structural alterations consist of gross disorganization of the seminiferous tubules, with detachment of the spermatogonial cells from the basal membrane and absence of spermatocytes. Results from both *in vivo* and *in vitro* studies have indicated that the Sertoli cell is the main target for DEHP-induced testicular toxicity and that MEHP is the ultimately active testicular toxicant [15, 18, 21]. The Sertoli cell is a somatic cell type whose integrity and functionality is required for the growth and maintenance of the germ cells as they divide and differentiate from spermatogonia to spermatocytes and ultimately to spermatids. The latter are released by the Sertoli cell into the lumen as sperm.

In recent years, concern has also been raised that many industrial chemicals, DEHP among them, are endocrine-active compounds capable of having widespread effects on humans and wildlife

[22, 23]. Particular attention has been paid to the possibility of these compounds mimicking or antagonizing the action of estrogen, and more recently, their potential anti-androgenic properties. Estrogen influences the growth, differentiation, and functioning of many target tissues, including female and male reproductive systems, such as mammary gland, uterus, vagina, ovary, testes, epididymis, and prostate. Thus far, however there is no evidence that DEHP is an endocrine disruptor in humans at the levels found in the environment.

Despite the above mentioned research we still do not understand the metabolism or toxic effects of plasticizers in detail, so the purpose of this study was to indicate which main metabolites are produced and in what quantities, by which specific cell lines and plasticizers, as well as their effect on the cells. The results could serve as a model to what might happen in the body when certain organs are exposed to plasticizers.

Methodology

Chemicals. Di(2-ethylhexyl)phthalate, Di(2-ethylhexyl)adipate, 2-ethylhexanol, myristic acid and Tetramethyl ammonium hydroxide (TMAH) and Tetramethyl phenyl ammonium hydroxide (TMPAH) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) at least 99% purity. 2-Ehtylhexanoic acid was purchased from Acros (Acros Organics, USA) while Chloroform, ethyl acetate, dimethyl sulfoxide, ethyl ether, phenolphthalein indicator and sulfuric acid were from Fisher (Fisher Scientific Company, Nepean, Ontario, Canada). Pentadecane was obtained from A&C chemicals (A&C Produits Chimiques Americain, St-Laurent, QC).

Cell Growth. Three mammalian cell lines were selected based on exposure to xenobiotics and availability. These were human umbilical vein vascular endothelium cells (HUVECC) ATCC CRL-1730 (pass 6-8), mouse hepatocytes cells ATCC CRL-2254 (pass 15-20), and human hepatocellular carcinoma cells (HepG2) ATCC HB-8065 pass 20-30. The media used were endothelial cell medium, low serum (Promocell, Heidelberg, Germany), Eagle's minimum essential medium with Earl's balanced salt solution (Fisher Scientific Company, USA), and HYQ DME/F-12 1:1 with 2.5 mM L-glutamine and 1.5 mM hepes buffer (Fisher Scientific Company, USA). All cell lines were grown using their ATCC recommended media supplemented with 10% v/v fetal bovine serum (Fisher Scientific Company, Nepean, Ontario, Canada) and 1% v/v penicillin-streptomycin preparation (Fisher Scientific Company, USA) in T-175 flasks (Fisher Scientific Company, USA) at 37°C with 5% carbon dioxide. Trypsinization and subculturing was done by washing the cells with 1X PBS, adding trypsin (Fisher Scientific Company, USA) and placing the flask an incubator for 15 minutes. The cells would subsequently be checked under the microscope and resuspended in media. Human endothelial cells would then need to be centrifuged and resuspended to remove all the trypsin since the medium as low serum. Reseeding was always done to a ratio of 1:4.

Cellular effect studies. The effect of the treatment was measured as the ability to enhance detachment of mouse hepatocytes from the adherent monolayer into the medium. Cells were subcultured into T-25 flasks until full confluency and the spent culture medium was replaced with 10 ml fresh medium containing 500 µM DEHA. DMSO was used to dissolve DEHA before addition to the media (final DMSO concentration 0.5% v/v). Control cultures received medium containing 0.5% v/v DMSO alone. Each flask was then observed under an inverted microscope at 10X magnification and three spots marked at random at the bottom of the flask. Pictures were then taken of the cells at these three spots, with a digital camera attached to the microscope, right after the treatment and after 2, 4 and 12 days. The magnification allows for about 1 mm^2 field of view and a cell count was manually performed for each of the pictures and averaged over the overall surface.

Esterase enzyme assay. The presence and activity of esterase enzymes was verified and quantified according to a method developed by Dominic Sauvageau (Department of Chemical Engineering, McGill University, Montreal, QC, Canada) with minor alterations. Cells were subcultured in T-25 and grown to confluence after which the spent medium was replaced with 10 ml of fresh serum-free medium containing butyl butyrate at 3 mM. The flask was then incubated for 30 minutes, after which the media was drawn out and extracted according to the procedure described below.

In vitro studies of metabolism. Confluent T-175 flasks were trypsinised with 2 ml of trypsin after the PBS wash to minimize concentrations of trypsin during the experiment. The cells were then resuspended in 50 ml of serum-free medium and centrifuged (Thermo Corporation Centra CL-2) at 1200 rpm for 5 minutes in a 12.1 cm rotor. The medium was subsequently removed leaving 10 ml for cell resuspension which would then be divided up in glass vials as 2 ml aliquots. This would give about $1x10^6$ cells/ml by hemocytometer count. The test compound was then added to a concentration of 500 µM, dissolved in DMSO as a solubilizing agent. DMSO concentrations were always kept at 0.5% v/v and control cultures contained 0.5% v/v alone. The vials were then sonicated at medium settings (Ney 300

Ultrasonik) for 15 minutes. For SPME samples the vial was sealed with parafilm (Fisher Scientific Company, USA) before placing the vial in the incubator. The vials were taken out of the incubator daily and frozen at -20°C for no more than 48 hours before extraction and analysis.

Sample extraction and analysis. Each sample is acidified with sulfuric acid to a pH of ~2. An equivalent volume of chloroform containing 0.1 ml/l pentadecane as internal standard is then added and the mixture vortexed for 1 minute. The sample is then left to stand for about 10 minutes before the bottom fraction is separated by syringe and centrifuged at 10,000 rpm for 2.5 minutes to obtain clear chloroform. This would then be transferred to a 1.5 dram vial and stored at -4°C for no more than 1 hour before injection in the gas chromatograph (GC) at room temperature. The gas chromatograph (HP5890 Series II) used a 15 m x 0.53 mm SIL-5CB column (Varian, St. Laurent QC, Canada). The settings of the GC were: injector temperature of 250°C, initial column temperature of 40°C, temperature ramp rate of 10°C/min until 150°C, the 20°C/min until a final column temperature of 250°C, detector temperature of 300°C, ramp hold time of 2.5 min, and final hold time of 0.1 min.

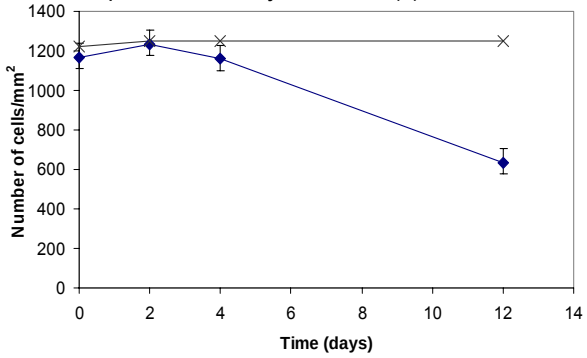
In the case of derivatization, the same procedure is executed but with ethyl acetate with 0.1 g/l myristic acid as internal standard instead of chloroform. The extract is then treated according to ASTM method S974-96 which roughly consists of blowing the sample down with nitrogen, re-suspension in ethyl ether, adding phenolphthalein indicator and adding TMAH drop-wise until a color change is seen. TMPAH was found unsuitable as it caused DEHA to chemically break down to adipic acid. The same GC was used but with column SPB-5 (Supelco). The settings of the GC were: injector temperature of 250°C, initial column temperature of 60°C, temperature ramp rate of 10°C/min until 130°C, then 50°C to the final column temperature of 280°C, detector temperature of 300°C, ramp hold time of 2.5 min, and final hold time of 0.1 min. SPME was also performed to analyze the presence of metabolites in the gas phase. The syringe was in the sealed vial for 10 minutes and transferred to the GC with the same derivatization column. The GC settings in this case were: injector temperature of 250°C, initial column temperature of 60°C, temperature ramp rate of 10°C/min, final column temperature of 1800°C, detector temperature of 300°C, ramp hold time of 2.5 min, and final hold time of 0.1 min.

Results and discussion

Cell detachment studies. With time in culture, mouse hepatocytes detached from the cell layer in to the culture medium. The addition of DEHA markedly accelerated this process of hepatocytes cells detachment. Cell detachment occurred in random patches, and this effect is more readily apparent after 4 days of culture, where the number of attached cells in medium containing DEHA starts to deviate from the control which just contained DMSO. After 12 days the control flask was still confluent since the medium added was in excess at 1220 cells /mm^2, while the experimental flasks had 650 cells/mm^2. The results of these experiments, done in triplicates, are shown in Figure 2.

As would be expected liver cells of both species human and mouse demonstrate higher esterase activities than endothelial cells. Human liver cells also show a slightly higher activity compared to their mouse counterparts, and were thus selected to be used for metabolism studies.

Figure 2. Number of mouse hepatocytes present in the adherent monolayer after addition of DEHA (♦) and in control experiments with just DMSO (x).



Esterase enzyme assays. Table 1 shows the activity in mM of butyl butyrate degraded per minute assuming that each T-25 flask contained approximately the same number of cells at confluence.

Table 1. Esterase enzyme activity of potential plasticizer exposed cells as millimoles of butyl butyrate degraded per minute.

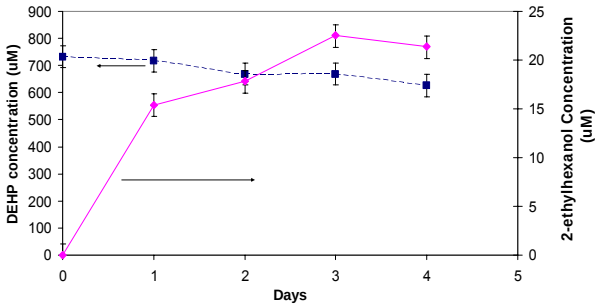
Cell Line	*Activity (mM/min)*
Endothelial cells	0.03
HepG2	0.06
Mouse Hepatocytes	0.05

In vitro studies of metabolism. A typical 5-day study of degradation by HepG2 cells in the presence of DEHP is shown in Figure 3.

Each data point was obtained as an average of triplicate experiments. DEHP decreases steadily from 720 µM to 620 µM over the five day period. A new peak was identified in the GC chromatograph between the solvent and the internal standard.

This compound appears before the plasticizer and was identified as a possible metabolite after comparison with abiotic controls.

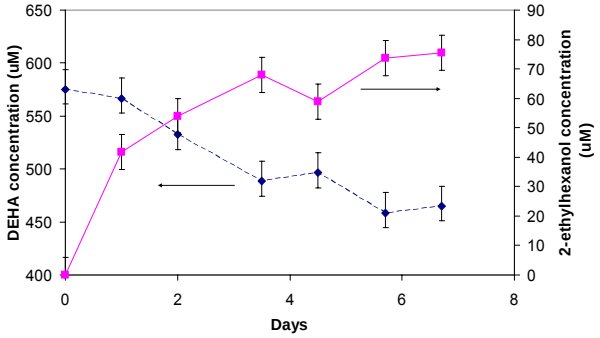
Figure 3. Concentrations of DEHP (■) and 2-ethylhexanol (♦), on the left and right axis respectively.



The concentration of this metabolite increased rapidly at first, then more slowly until it seemed to stabilize at about 22 µM after 3 days.

A similar trend was observed with DEHA and a peak having the same retention time was also seen. The data for five replicate experiments involving the addition of DEHA to HepG2 cells is shown in Figure 4. The rate at which the metabolite concentration increased was much faster and after 5 days the concentration in the medium was about 75 µM and seemed to be stable, as was the case with DEHP. In this case the concentration in DEHA also dropped from 570 µM to 470 µM but seemed to stabilize during the last two days. The abiotic vials did not show any new peaks, and the concentration remained the same as the initial concentration.

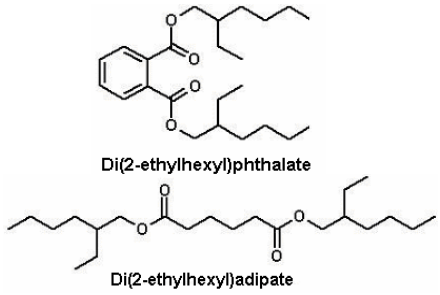
Figure 4. Concentrations of DEHA (■) and 2-ethylhexanol (♦), on the left and right axis respectively.



The structure of the two compounds may explain the differing rates of 2-ethylhexanol production seen in Figures 3-4. A case for stearic hindrance can be made as the efficiency of hydrolysis of DEHP suffers from the presence of both the two side chains being close to one another and the benzene ring next to them as seen in Figure 5. Further experiments with other plasticizers would be needed to confirm this explanation.

The unknown peak was hypothesized to be 2-ethylhexanol, 2-ethylhexanal or 2-ethyhexanoic acid from the available literature although 2-ethylhexanal is a very volatile chemical.

Figure 5. Structures of DEHP and DEHA [1].



Addition of each chemical to samples of the chloroform extraction as well as retention time comparison confirmed that the peak observed is due to the presence of 2-ethylhexanol. The same chemical is also seen in SPME experiments where the only peak present matches with the peak formed when the syringe is exposed to 2-ethylhexanol vapors, and the gaseous concentration is estimated to be about 4% of the liquid molar concentration (results not shown). The monoester was not seen but it is hypothesized that it is mainly in the form of the glucuronic acid conjugate and thus very water soluble and hard to extract into chloroform. Adipic acid was found after derivatization confirming that some of the 2-ethylhexanol is a results of the hydrolysis of the monoester. There were thus no peaks from 2-ethylhexanal or 2-ethylhexanoic acid in both studies with DEHP and DEHA which contradicts bacterial and some of the mammalian metabolism literature. Other experiments outlined in Table 2 were thus carried out to test whether 2-ethylhexanal and 2-ethylhexanoic acid are so rapidly metabolized as to not appear in the extract.

Table 2. Concentrations of DEHA, 2-ethylhexanol (2-ehol) and 2-ethylhexanoic acid (2-ehoic acid) in HepG2 medium after 3 days.

Initial Concentration	DEHA (µM)	2-ehol (µM)	2-ehoic acid (µM)
500 µM 2-ehoic acid	0	0	500
50 µM 2-ehoic acid	0	0	50
500 µM DEHA + 50 µM 2-ehoic acid	411	55	50
500 µM 2-ehol + 50 µM 2-ehoic acid	0	84	50

HepG2 cells do not seem to be able to degrade 2-ethylhexanoic acid at high (500 µM) or low (50 µM) concentrations and this does not seem to be due to a lack of enzyme induction effect since when DEHA or 2-ethylhexanol are added together with 50 uM of 2-ethylhexanoic acid, the acid does not get metabolized at all. This suggests that 2-ethylhexanoic acid is indeed not degraded by this

cell line and so it cannot be produced since it does not appear in the chromatograph during plasticizer degradation.
Thus the HepG2 cell line does not seem to be able to degrade the plasticizers DEHP and DEHA beyond 2-ethylhexanol and so the pathway obtained is that shown in Figure 1 but without 2-ethylhexanal and 2-ehtylhexaoic acid. This could be due to two factors: stearic hindrance or the lack of the enzyme alcohol dehydrogenase. In fact the ethyl branch in the 2 position could be obstructing the action of the enzyme as is the case with many bacteria [18] and branched hydrocarbons. The alternative to that is of course that alcohol dehydrogenase is not produced at all in this cell line, but rather in another part of the body, which is a possible downside of comparing data from a single cell line to data from the myriad of cell lines forming a whole mammalian organism.
It was also found that human endothelial cells and mouse hepatocytes were also able to metabolize DEHA. All three cell types tested thus seemed to posses the ability to degrade plasticizers to a certain extent. This was also seen when the cells were not centrifuged prior to incubation although in that case the rate of 2-ethylhexanol production was slight lower (results not shown). The apparent small increase in concentration of these enzymes after sonication however seems to suggest that the enzymes that cause the hydrolysis of the ester bonds are intracellular, which agrees with the data from Carter et al. [14] in that most of the hydrolysis takes place in the liver mitochondria and microsomes, but not in the cytosol.

Conclusion

Plasticizers are known to enter the environment as they leach from plastic products and migrate by means of water and air transport. This will ultimately ensure that humans and other animals will be exposed to them as they enter the body. Research has shown that they can be metabolized in the mammalian organism, but the details are not yet fully understood. This work has shown that human umbilical vein vascular endothelium cells, mouse hepatocytes cells and human hepatocellular carcinoma cells are capable or metabolizing plasticizers to the final products 2-ethylhexanol and adipic acid. These results however differ from previous reports in that 2-ethylhexanal and 2-ethylhexanoic acid are not seen as 2-ethylhexanol breakdown products, either due to stearic hindrance effects or the lack of enzymes in the specific cell line. Plasticizer di(2-ethylhexyl)adipate was also found to affect cell growth as it enhances cell detachment from the adherent monolayer into the culture medium compared to control experiments.

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