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INTERACTION OF DEHA WITH MAMMALIAN CELLS

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Abstract

This project studied the biodegradation of a plasticizer, di-(2-ethylhexyl) adipate (DEHA), by two mammalian cell lines, HepG2 and WIF-B, *in vitro*. An MTT assay showed that DEHA had a toxic effect on both cell lines. Despite this, both hepatocyte cell lines successfully degraded the plasticizer. Metabolites were identified and quantified by gas chromatography. HepG2 cells showed minimal alcohol dehydrogense activity and this resulted in the accumulation of 2-ethylhexanol. WIF-B cells were able to breakdown the alcohol and produced 2-ethylhexanoic acid. It is important to note that an enzyme was essential for this step in the degradation of the plasticizer, as this proves that it was biodegradation and not physical degradation. By comparing the metabolites formed and the order of their appearance, the degradation pathway in these mammalian cells was found to be similar to the established degradation pathways for bacteria, fungi and yeast.

Sommaire

Ce projet a étudié la biodégradation *in vitro* d'un plastifiant, DEHA, par deux lignées de cellules de mammifères: HepG2 et WIF-B. L'analyse MTT a démontré que le DEHA avait un effet toxique sur les deux lignées de cellules. Néanmoins, ces hépatocytes ont réussi à dégrader le plastifiant. Les produits de cette métabolisation ont été identifiés et quantifiés par la chromatographie en phase gazeuse. Les cellules HepG2 ont démontré une faible activité d'alcool déhydrogenase et par conséquence, une accumulation du 2-éthylhexanol s'est produite. Les cellules WIF-B ont été capable de métaboliser cet alcool et produire de l'acide éthyl-2 hexanoïque. Il faut noter que l'utilisation d'une enzyme a été nécessaire pour arriver à la dégradation du plastifiant et ainsi mettre en évidence la biodégradation de ce plastifiant et non la dégradation physique de celui-ci. En comparant les métabolites produits et leur ordre d'apparition, on a démontré que la voie de dégradation du plastifiant dans les deux lignées de cellules de mammifères était similaire à celle de bactéries, champignons et levures.

Acknowledgments

First and foremost, I would like to thank my supervisors, Dr. David Cooper and Dr. Richard Leask for their support and guidance with this project. It would not have been possible without their insight or encouragement. Thank you to Pira Korsiporn who started this project in our labgroup and set up the methods. I would also like to acknowledge Ranjan Roy for his help troubleshooting the GC and for teaching me more than I ever thought I could know about "Frankenstein"! Thanks to Dom T. and Hervé for correcting my French abstract, as well as to all the other students in both lab groups for their help and friendship: Sarah, Dom S., Azadeh, Nic, Helen, Joanna, Stacey, Leonie, Jeff, David and especially my wonderful officemate Tiffany.

Table of Contents

Figures

Tables

1 Introduction

This project investigates the degradation of di-(2-ethylhexyl) adipate (DEHA), by mammalian cells. The metabolism of plasticizers by bacteria is well known[1, 2] and some of the produced metabolites have been shown to be more toxic than the parent compound. The metabolism of plasticizers by mammals is poorly understood but of real concern as plasticizers can be absorbed in the body through the digestion, inhalation or contact with the skin[3-5].

1.1 Plasticizers

The plastics industry is the fourth largest manufacturing industry in the U.S. In 2005, industrial shipments were valued at $341 billion dollars[6]. Integral to the production of plastics, are additives called plasticizers. These are low molecular weight molecules added to improve the properties of the plastic. They reduce the glass-transition temperature of the plastic to facilitate its processing and/or its final properties. The plasticizer is embedded between the polymer chains of the plastic and is not chemically bonded to it. As a result, plasticizers have been shown to leach out of the plastic in large quantities over time and accumulate in the environment[4, 5].

The most common plasticizer is di-(2-ethylhexyl) phthalate, (DEHP), which is produced at a rate of 500 thousand tons per year[7] (Figure 1.1). It is predominantly used in the manufacturing of polyvinyl chloride (PVC). It is

present in construction materials, car products, cling wrap, toys, blood storage bags and medical devices[5]. DEHP can represent between 20 and 40% of the final weight of the plastic[8]. DEHP belongs to a family of phthalate ester plasticizers. Another common plasticizer is di-(2-ethylhexyl) adipate (DEHA) (Figure 1.1). This is found primarily in PVC films used as plastic food wrap. This plasticizer has been found at a high concentration in Montreal waste water[9].

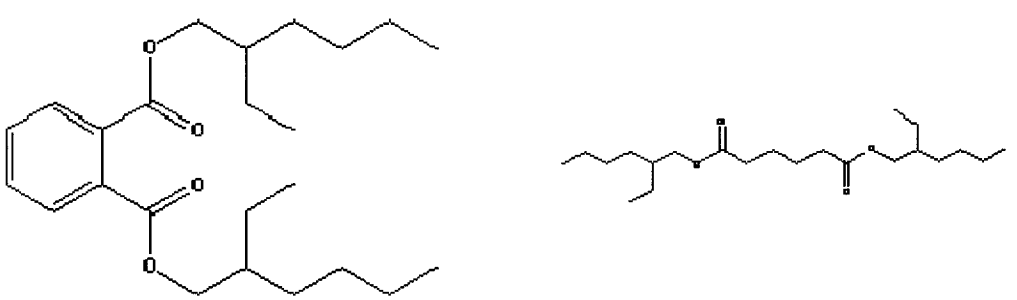

Figure 1.1 Structures of DEHP (left) and DEHA (right)

1.2 Environmental Consequences

Plasticizers leach out of the polymer matrix, both during useful life of the plastic and after its disposal. As a result, plasticizers have become persistent contaminants in the environment. There have been studies examining the breakdown of plasticizers by bacterial cells, yeast and fungi [1, 10-12]. The pathway of DEHA degradation (Figure 1.2) is well defined for bacteria, yeast and fungi and generates some harmful products during metabolism.

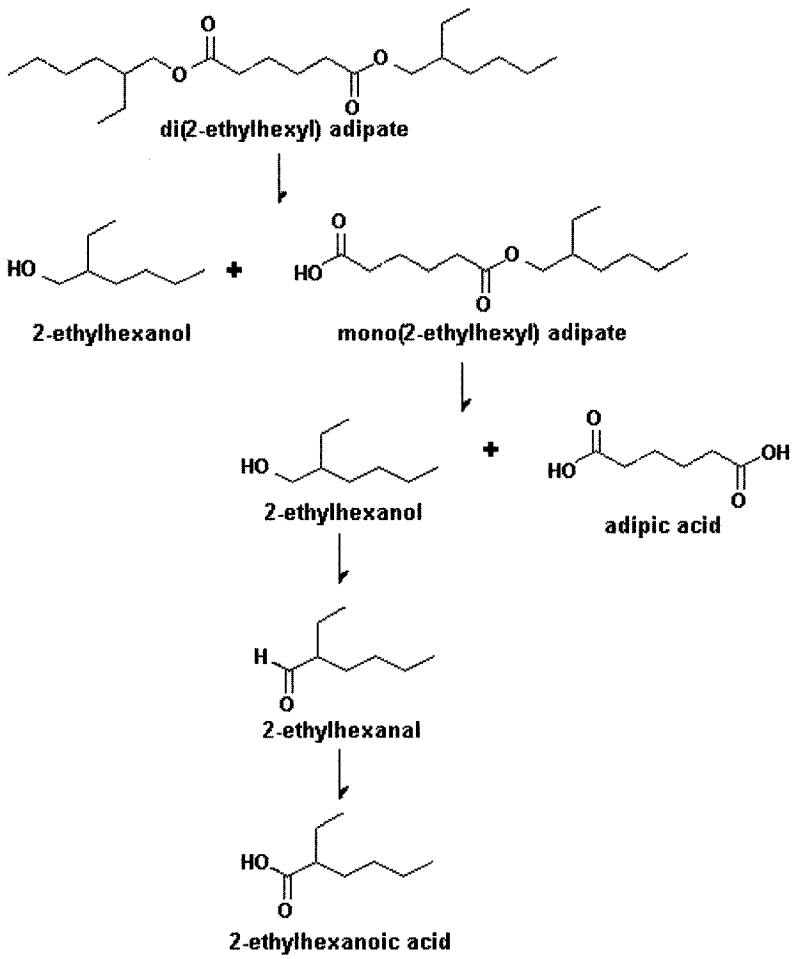

Figure 1.2 Degradation pathway of DEHA in bacteria, yeast and fungi[2]

DEHA breaks down to 2-ethylhexanol and 2-ethylhexanoic acid, both of which have been shown to be more toxic than the plasticizer. Acute toxicity is often assessed using the LD_{50}, which is a measure of the dose that is lethal to 50% of the test population, usually of rats. As demonstrated in Table 1.1 below, the two metabolites are almost 3 times more toxic than DEHA itself.

Compound	LD_{50} rat
DEHA	9100 mg/kg
2-ethylhexanol	3730 mg/kg
2-ethylhexanoic acid	3000 mg/kg

Table 1.1 Toxicity of DEHA and its metabolites in rats[13-15]

1.3 Mammalian Exposure

People are also exposed to plasticizers on a daily basis, through inhalation and ingestion of water and food[3-5]. It is also important to note that daily exposure to DEHP may be three times higher for people in a clinical setting than for the general population[16]. The mammalian degradation of plasticizers is believed to be similar to that observed in lower organisms; however, the mechanism and location of this metabolism is still not apparent.

In mammals, the breakdown has been studied in the liver, lungs, kidneys and intestines[17], *in vivo*. These studies have all shown the ability of mammalian systems to partially degrade plasticizers. However, the pathway is difficult to resolve *in vivo*. *In vitro* studies are limited by the fact that cultured cells lose cell-specific functionality, and some or all enzymatic activity, making plasticizer metabolism difficult to investigate fully.

This study attempts to overcome some of these difficulties by using two different cell lines, one of which (WIF-B cells) has enhanced enzymatic activity,

designed to mimic *in vivo* activity. This cell line, developed by the Hubbard Laboratory[18] has been used to investigate liver function and interactions with alcohol[19-21]. This will help establish the importance of enzymes in the degradation and in indicate whether biodegradation or physical degradation is responsible. Gas chromatography is used to identify and quantify the metabolic products. The toxicity of the plasticizer and metabolites as well as the effect on cell viability are also investigated. These experiments seek to further the understanding of plasticizer metabolism in mammalian systems.

2 Objectives

The purpose of this project was to investigate the fate of plasticizers in mammals. These experiments sought to complement existing literature assessing the risks of plasticizers to humans. This is especially important due to the continued and increased use of plasticizers in industry and their resulting accumulation in the environment. The interaction of plasticizers in human systems is largely unknown but is important, especially for people who are chronically exposed to them.

It was hypothesized that mammalian cells are capable of DEHA metabolism and that the pathway is similar to that which has been established for bacteria. The metabolism of DEHA was examined using two different cell lines, to prove that it was biodegradation and not physical degradation.

To study the degradation of DEHA by mammalian cells, experiments were performed *in vitro*. The experiments were done to determine if DEHA is inert in mammalian systems or if it is degraded. By comparing the metabolites formed, the degradation pathway in mammalian cells were compared to known degradation pathways in bacteria, fungi and yeast[2]. Differences in rate and degree of degradation were compared between distinct mammalian cell lines and between mammalian cells and bacteria. Additional experiments investigated the toxicity of the plasticizer and its metabolites on mammalian cells.

Specific objectives of the study were to:

- Study the biodegradation of DEHA in mammalian cells, *in vitro*
- Investigate the effect of DEHA on cell viability
- Identify and quantify the metabolites
- Examine the importance of alcohol dehydrogenase (ADH) in DEHA metabolism

3 Literature Review

3.1 Plasticizer Degradation

The biodegradability of many different plasticizers have been studied in different environments, including waterways, activated sludge, hydrosoil and shake flasks[1]. The relative biodegradability of these compounds has been investigated as well as their respective metabolites. In particular, the biodegradation pathway for DEHA by bacteria, yeast and fungi has been described[2]. DEHA is partially hydrolyzed to produce 2-ethylhexanol and mono(2-ethylhexyl) adipate (MEHA), which is then hydrolyzed to release 2-ethylhexanol and adipic acid. The alcohol is oxidized to 2-ethylhexanal and then to 2-ethylhexanoic acid (Figure 1.2).

3.2 Plasticizer degradation in mammalian cells in vivo

Research into mammalian plasticizer degradation was sparked when it was discovered that plasticizers were leaching from blood storage bags into the blood[22]. The first experiments were published in 1973 by Albro and Fishbein[23]. In later studies, the metabolites were investigated; however, unlike bacterial degradation[2], neither the acid nor the alcohol was found. It was postulated that these may avoid detection if they are quickly metabolized.

The use of DEHP in medical equipment is widespread. It can be found in bags for blood, plasma, intravenous fluids, total parenteral nutrition, tubing for the

administration of these fluids, umbilical catheters, extracorporeal membrane oxygenation (ECMO) circuit tubing, hemodialysis tubing, and examination gloves[24]. Green *et al.* studied the DEHP exposure on neonatal intensive care infants by monitoring their urine. They observed a direct association between the amount of DEHP exposure and the quantity of MEHP in the urine. In laboratory animals, MEHP produces developmental, reproductive and hepatic toxicity[5]. These studies have demonstrated that mammals are able to break down common plasticizers.

DEHP degradation by different mammalian species was studied by Ito *et al*[25]. They homogenized the lung, kidney, intestine and liver tissue of mice, rats and marmosets and exposed samples to the plasticizer. These organs were studied because the lungs and intestine are known to absorb DEHP, the liver is known to metabolize DEHP and the kidney has been shown to excrete DEHP. The activities of the four enzymes involved in metabolizing DEHP were studied: lipase, UDP-glucuronyl transferase (UGT), ADH and ALDH. DEHP is hydrolysed to 2-ethylhexanol and MEHP by the catalytic action of lipase. Some MEHP is excreted in the urine after it is conjugated with UDP-glucuronide by UGT[26]. MEHP can also be oxidized by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH) to dicarboxylic acid or ketones[26]. Remaining MEHP is excreted unchanged in the urine. 2-Ethylhexanol is oxidized to 2-ethylhexanoic acid via 2-ethylhexanal by ADH and ALDH. These metabolites are similar to those observed in DEHA degradation.

The biggest difference between the species was in lipase activity. This was much higher (150 to 350 fold) in mice than in marmosets. This suggests that DEHP is hydrolysed in rodents much more easily than primates. UGT and ADH activity was observed in all three species and the highest level was in the liver. ALDH was measured in all three species, though the level in mice was much lower than marmosets[25]. The level was highest in the small intestine and kidney of marmosets; however, in mice, it was not seen in these organs. It was shown that ADH and ALDH activities are higher in primates than in common experimental mammals, mice and rats. These differences seen between the species suggest that caution should be observed when extrapolating results from rodents to humans.

At the focal point of this thesis is DEHA, the plasticizer commonly found in plastic food wrap. If DEHA leaches from the plastic, it can be absorbed into the packaged food. Studies have measured the amount of DEHA that migrates into wrapped food. One study in particular, reported that DEHA in PVC (5.3% w/w DEHA) migrated into halawa tehineh (halva, sesame paste) samples. After 240 hours the level of DEHA in the halva samples was 81.4 mg/kg halva, which corresponds to a loss of 54.7% (w/w) DEHA from the plastic[27]. DEHA has also been detected in commercially packaged food products, including a study which looked at levels in curry paste[28] and found concentrations ranging from 4.0 ng/g to 0.61 μg/g. As a result of this contamination in food it is not surprising that DEHA and other plasticizers are present in humans. The daily intake of DEHA

from food was found to be 2.2 mμg/kg body weight[29]. From these studies, it is evident that DEHA can be absorbed into the human body. *In vitro* experiments have also been conducted to better understand the effect of plasticizers in mammalian systems and to better define the metabolic products.

3.3 In vitro degradation of plasticizers in mammalian cells

Most *in vitro* studies have focused on hepatocyte metabolism as it is believed that the liver is the primary site of plasticizer degradation. It is important to establish an appropriate model of mammalian liver cells for *in vitro* applications. Unfortunately, it is well documented that hepatocytes lose the ability to express ADH and are incapable of effectively metabolizing alcohols as well as losing other liver-specific functions[30-33]. This is problematic for plasticizer degradation, as an alcohol is one of the metabolites. Without ADH, the alcohol accumulates in the culture media simulating chronic alcoholism. Liver damage in alcoholic patients results from both nutritional factors and of a direct toxic effect of ethanol in the liver[34]. This damage effects the liver at a cellular level, changing the metabolite pattern, redox states, phosphorylation potential[35] and other metabolic changes, some of which have been shown to be detrimental to the cells[36].

In order to study metabolism *in vitro*, different cell lines have been developed which retain the ADH enzyme. The use of recombinant clones is a popular method. HepG2 cells can be transfected with recombinant plasmids

carrying an ADH gene and have been shown to exhibit significant ethanol oxidation to the aldehyde[19, 37-39]. One line, WIF-B, is a highly differentiated and polarized rat hepatoma/human fibroblast hybrid[31]. This cell line is stable and exhibits many hepatocyte-specific phenomena[31]. In experiments, WIF-B cells have been shown to have approximately twice the ADH activity of freshly isolated hepatocytes and the ethanol concentration in cell cultures decreased linearly with time[31]. These cells are an appropriate model for investigating the morphological and functional status of hepatocytes exposed to ethanol. Therefore this cell line has been chosen to better understand the metabolism of plasticizers *in vitro*.

4 Materials and Methods

4.1 Chemicals and Media Components

A complete list of chemicals used appears in Appendix A. Chemicals were all reagent grade and were sterilized by autoclaving (AMSCO model 3021-S) for 30 minutes at 121°C. In cases where the temperature of the autoclave risked changing the structure of chemicals, or if only a small quantity was needed, they were filtered at 0.22 μm (Appendix A).

4.2 Mammalian cells

Two cell lines were used: HepG2 and WIF-B. HepG2 cells are human hepatocellular carcinoma cells and were obtained from ATCC (HB-8065). WIF-B cells were a generous gift from Dr. Ann Hubbard at John Hopkins University. These two cell lines were chosen because the liver has been shown to be the primary location of plasticizer metabolism. HepG2 are commonly used liver cells; however, they have been shown to exhibit little alcohol metabolism in culture. A more appropriate model for human liver cells are WIF-B cells, which have been transfected with an additional alcohol dehydrogenase gene.

4.3 Cell Culture

Mammalian cell culture requires the simulation of *in vivo* conditions. To meet these specifications, the cells were cultured and expanded according to

established protocols, tailored to the laboratory's facilities. Biosafety level 1 protocols were observed (Appendix C).

Cells were grown in T-flasks (Fisher Scientific, Montreal, QC) with the appropriate media. The cells were incubated (Thermo Electron Corporation, Model Forma Series II Incubator, Montreal, QC) at 37°C with 5% carbon dioxide, as a gaseous buffer to maintain the pH of the media.

If contamination was suspected, as a result of dead cells, cloudy media, or a change in media colour, the media was plated on 2 brain heat infusion (BHI) agar plates. One plate remained at room temperature and the other was incubated at 30°C. Both plates were left for at least a week and inspected for bacterial growth.

4.3.1 Media Specifications

The recommended media for HepG2 cells by ATCC was Eagle's Minimum Essential Medium with Earl's balanced salt solution (Fisher Scientific, Montreal, QC). WIF-B cells were cultured with F-12 Kaign's Modification (Gibco/Invitrogen, Montreal, QC), which was supplemented with Glucose, Ascorbic Acid, Linoleic Acid, Amphotericin, HAT and Glutamax, according to protocols defined in Appendix B. Both cell culture media were supplemented with 1% v/v penecillin-streptomycin (Invitrogen, Montreal, QC) and 10% fetal bovine serum, FBS (Invitrogen, Montreal, QC).

4.3.2 Trypsinization

All cell culture manipulations were performed in a laminar flow fume hood (Thermo Electron Corporation, Model Class II, A2, Montreal, QC) and sterile conditions were maintained. The cell culture media was changed twice each week. Cells were trypsinized approximately once a week, when they were confluent in the flasks. The cells were first rinsed in 1XPBS and then trypsin (Fisher Scientific, Montreal, QC) was added. Cells were incubated with trypsin for 5 minutes, to allow them to detach. The cells were split and reseeded into two new flasks and media was added. An inverted optical microscope, (Leica DM IL, Leica Microsystems), was used to verify cell confluence, successful cell detachment after trypsinization and general cell health.

4.3.3 Cryopreservation

Cryopreservation was used to preserve cells before and after experiments. To thaw cells, they were removed from the cyropreservation tank and placed in the incubator. When thawed, new media was added slowly, to reduce osmotic shock and cells were reseeded in a T-flask.

To freeze a confluent T-flask of cells, ($1x10^6$ cells/mL), it was trypsinized and the contents were centrifuged at 1200 rpm for 5 minutes. The cells were then slowly suspended in a freezing medium of 10% v/v DMSO, 20% v/v FBS and 70% v/v media. This mixture was placed in a 1.5mL cryopreservation vial (Fisher Scientific, Montreal, QC). The vial was placed in an ethanol bath for at least 8

hours, in a freezer (Thermo Electron Corporation, Model forma -86°C ULT freezer, Montreal, QC) at -80°C. After this, the vial was placed in the cryopreservation tank.

4.3.4 Cell Quantification

To measure the cell concentration, 100 μL of suspended cells, at a concentration $1x10^6$ cells/mL, was added to 20mL of salt solution. Cells were counted using a Coulter Counter (Beckman Coulter Z2 Coulter Particle count and Size analyzer) and replicate counts were performed.

4.4 Cell Viability Assay

An MTT assay was done to evaluate cell proliferation. This laboratory test quantified cell growth based on colour change. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) oxidised to purple formazan and this change was measured with a spectrometer. Formazan was produced when reductase enzymes were active in the mitochondria of viable cells. This determined the amount of metabolically active cells. This assay can also be used to test drug sensitivity, cytotoxicity, response to growth factors and cell activation.

MTT experiments were performed in 96-wells plates. In order to measure the toxicity of DEHA on the cells, the plasticizer was added to the desired

concentration, usually 500μM, in the media. The cells were cultured in this media in the wells of the plate, with 0.5% v/v DMSO for at least 24 hours.

To begin the analysis, 10% v/v MTT was added to the cell culture and left in the incubator for 3 hours. One line of wells, without cells, was used as a control. Formation of formazan was confirmed using a microscope, and 100% v/v of detergent was added to lyse the cells and solubilize the coloured crystals. The samples were covered and left for another 3 hours in the incubator. The absorbance of the solution was read at 570 nm in a UV/Vis spectrophotometer (Cary 100 Bio) and in a Benchmark Plus plate reader (Bio-Rad). The amount of colour produced was directly proportional to the number of viable cells.

4.5 Plasticizer Experiments

To study the degradation of DEHA, the plasticizer was added to the media of the mammalian cells. DEHA was solubilized in di-methyl sulfoxide (DMSO) at a concentration of 0.5% v/v. Cells were trypsinized and media was added, containing 500μM DEHA, which is a concentration equivalent to that observed in human blood[24]. 2mL of cells and media was put into each 1.5 dram sample vial (Fisher Scientific, Montreal, QC) and the vial was capped. The samples were sonicated for 15 minutes at 200W (Neytech, model 300 Ultrasonik Broomfield, CT, USA) to break up cell agglomerations and they were returned to the incubator. At specific time intervals, between 0 and 8 days, samples were

removed and frozen at -20°C. The samples were thawed and then analyzed for plasticizer and metabolite concentrations by gas chromatography.

When studying the degradation of 2-ethylhexanol, the alcohol was added to the media at the desired concentration, usually 200 μM, solubilized in DMSO (0.5% v/v). The same procedure as above was followed for sonication, sample removal and analysis.

4.6 Analysis of Liquid Phase Metabolites

The media were analyzed for metabolites using gas chromatography. In order to prepare the samples for analysis, each 2mL sample was thawed and acidified by adding 5μL of sulphuric acid to protonate any organic acids. An extraction was then done by adding 2mL of chloroform, containing 0.1mL/L pentadecane as an internal standard and mixture was vortexed for 1 minute to ensure good mixing. The samples were allowed to settle for 10 minutes, to allow for a complete separation of the organic and aqueous phases. The bottom, organic, fraction was removed and placed into a microcentrifuge tube (Fisher Scientific, Montreal, QC). The sample was centrifuged at 10 000rpm for 2.5 minutes to obtain a clear liquid. This sample was then transferred to another microcentrifuge tube before being injected into the gas chromatograph. Samples were stored at -4°C for no more than 24 hours before analysis.

Gas chromatograph injection volumes were 10 µL, using a 10 µL syringe. The gas chromatograph used was a Varian model CP 3800, with a 30m x 0.32mm silica column SP SIL 8CB (Varian, St. Laurent QC, Canada). Initially, the settings of the gas chromatograph were: 1:10 injection split, an injector temperature of 250°C, a column temperature of 40°C and a detector temperature of 300°C. The column temperature had a ramp rate of 10°C/min until 150°C and then 20°C/min, until a final column temperature, 250°C was reached. There was a ramp hold time of 2.5 minutes and final hold time of 0.1 minutes.

For each compound investigated, a calibration curve was prepared by comparing the area of the peak of interest to the area of the peak of pentadecane, the internal standard. A linear relationship was established between this area ratio and the concentration of the compound of interest. In this way, the concentration of the compound of interest was determined in unknown samples.

4.7 Identification of Unknown Metabolites

New peaks appearing in the gas chromatographs were investigated as potential metabolites. The retention time was compared with the retention time of pure chemicals, run on the gas chromatograph using the same method. If a match was found, a small amount of the pure chemical was added to the original sample and run on the gas chromatograph again, to verify that the retention times were indeed the same and that no new peaks were observed.

5 Results

5.1 Analysis by Gas Chromatography

The samples from each experiment were analyzed using gas chromatography and a typical gas chromatograph is shown in Figure 5.1 below.

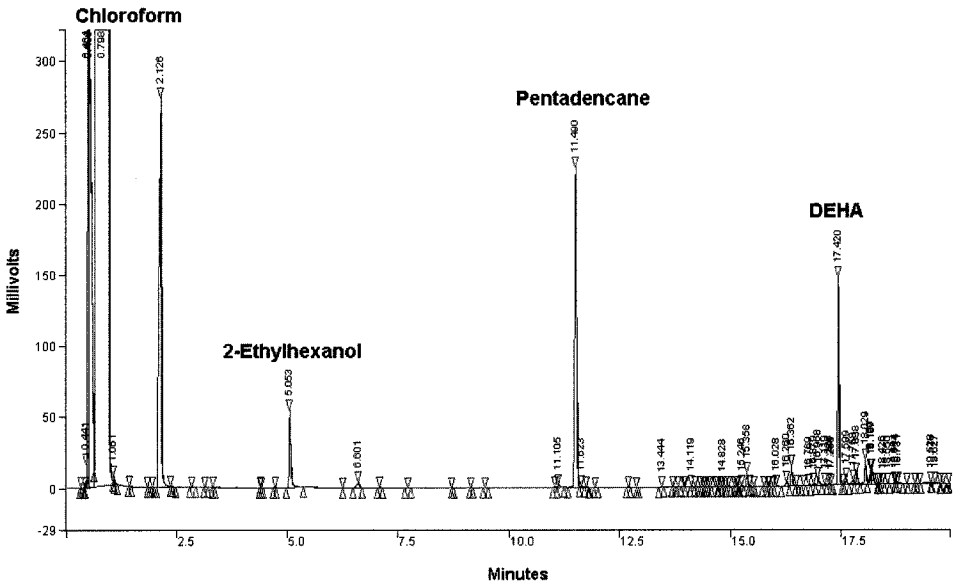

Figure 5.1 Typical Gas Chromatograph, Varian CP 3800, FID detector, column CP SIL 8CB

A calibration curve was prepared for each peak of interest.

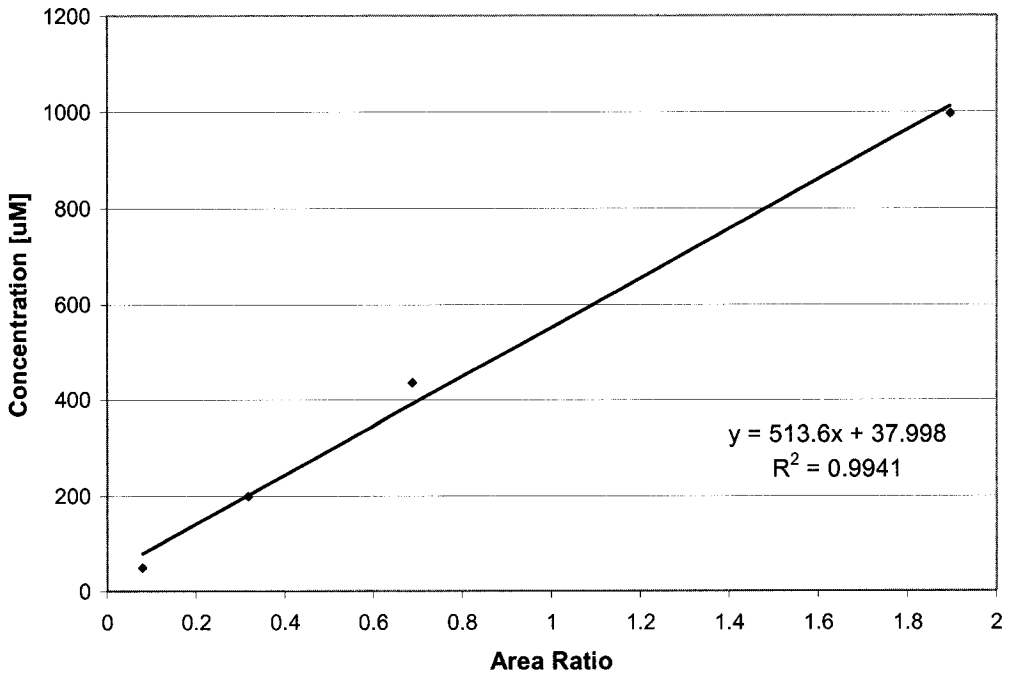

Figure 5.2 DEHA calibration curve

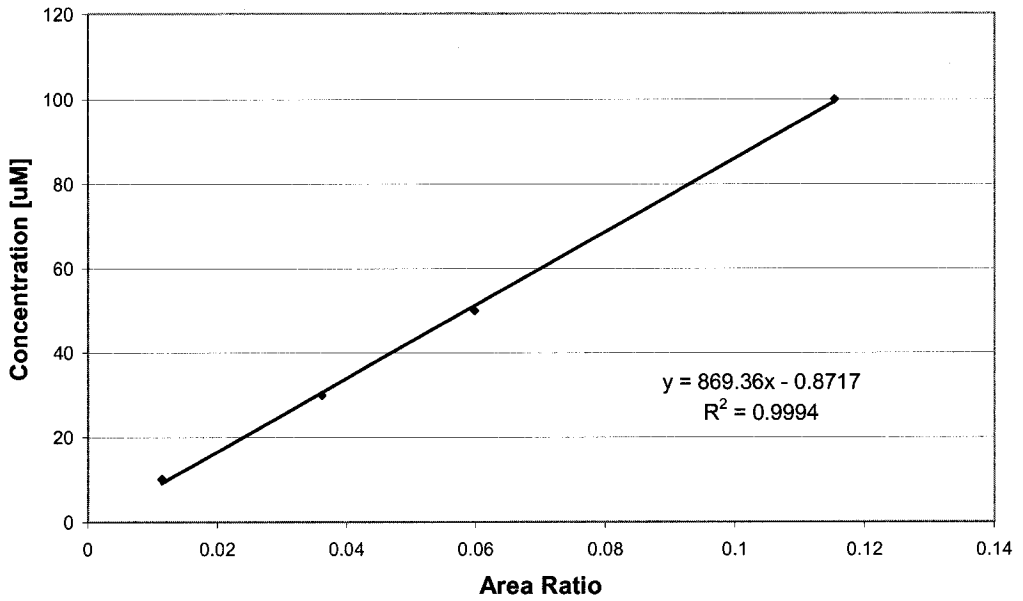

Figure 5.3 2-Ethylhexanol Calibration curve for 10 to 100 uM

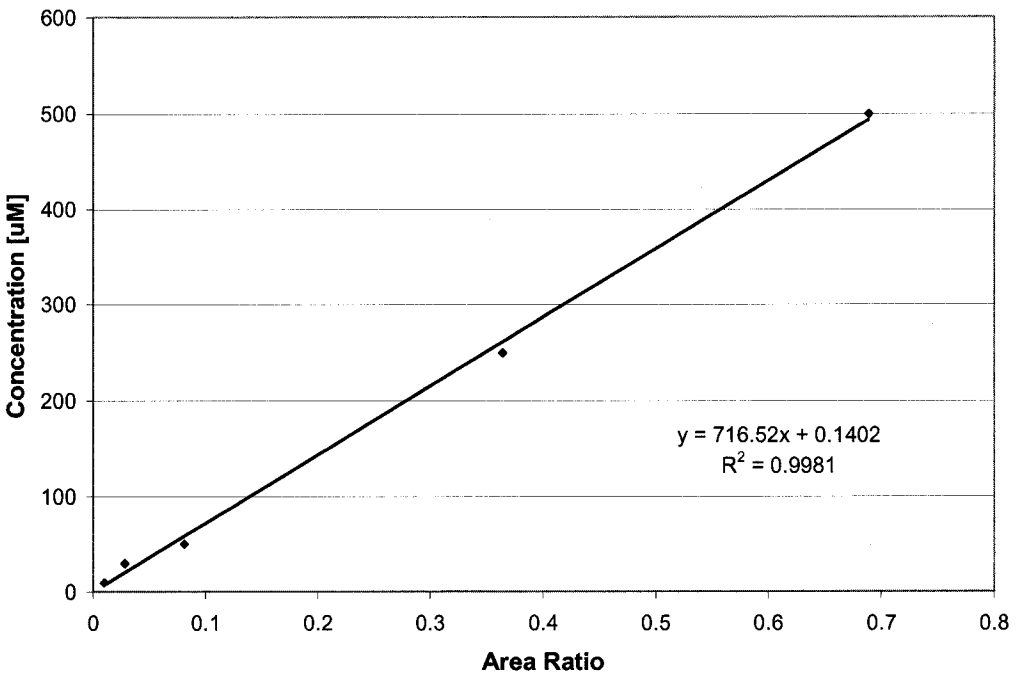

Figure 5.4 2-Ethylhexanol Calibration Curve for 100 to 500 uM

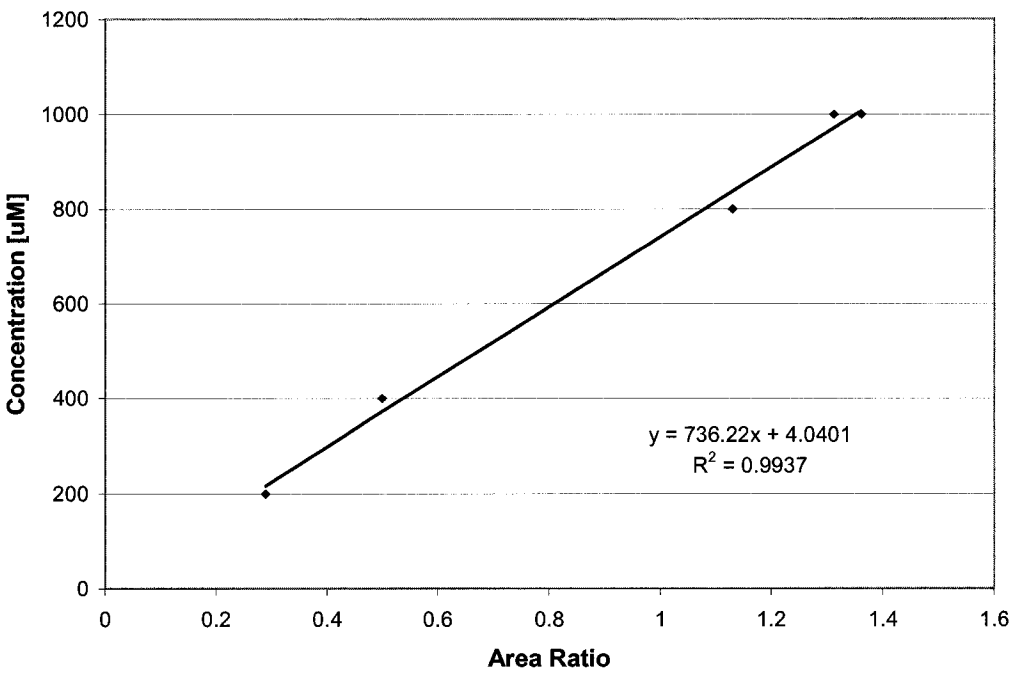

Figure 5.5 2-Ethylhexanoic Acid Calibration Curve

5.2 Cell Viability Assay

An MTT assay was done to test the toxic effect of DEHA on the cells. DEHA was added to the cells at a concentration of 500μM, in 0.5% v/v DMSO. In the control, 0.5% v/v DMSO was added to the cells, without DEHA. Cells were seeded at a concentration of $1x10^5$ cells/mL in a 96 well plate, containing 100μL of cell culture media. The media contained sufficient nutrients so that media changes were not required throughout the experiment.

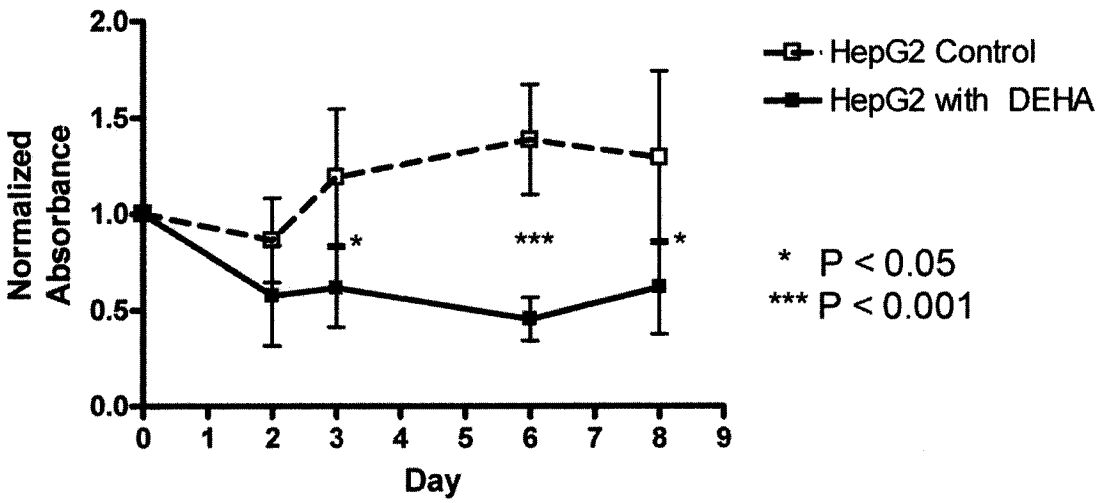

Figure 5.6 Normalized Absorbance of HepG2 cells with and without DEHA (n=3, bars indicate standard deviation)

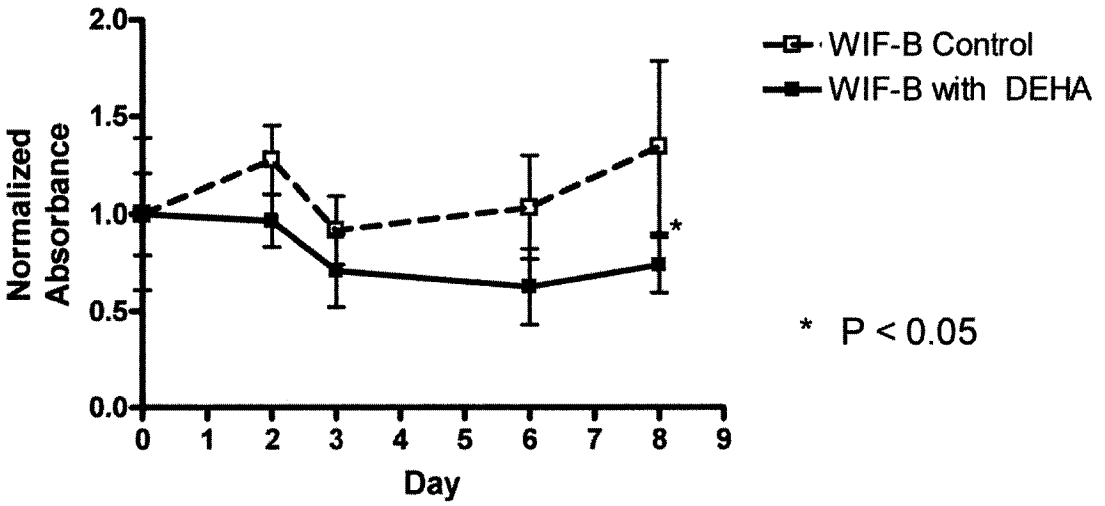

Figure 5.7 Normalized Absorbance of WIF-B cells with and without DEHA (n=3, bars indicate standard deviation)

As demonstrated in Figure 5.6 and Figure 5.7, HepG2 and WIF-B control samples continue to grow and proliferate. However, the cells with DEHA added showed a decrease in the number of metabolically active cells over time. Using a two-way anova, there was significant difference between the cells with plasticizer and without. The day was not significant but interaction of these variables did have an effect. The differences between the cells in the control experiment and with plasticizer present were statistically significant, especially at the later data points. For HepG2 cells, the difference between the control and the cells with plasticizer was significant as early on as day 3, ($P<0.05$, Bonferroni post test). The difference in viability is present at all following data points ($P<0.001$ at day 6 and $P<0.05$ at day 8, Bonferroni post test). WIF-B cells only showed a difference at day 8, ($P<0.05$, Bonferroni post test). However, there is a clear decreasing trend in the number of healthy cells for both cell types.

5.3 Monitoring DEHA

In order to further investigate the effect of DEHA on cells, cells were seeded at $1x10^6$ cells/mL and exposed to DEHA at a concentration of 500μM, in 0.5% v/v DMSO to identify metabolites.

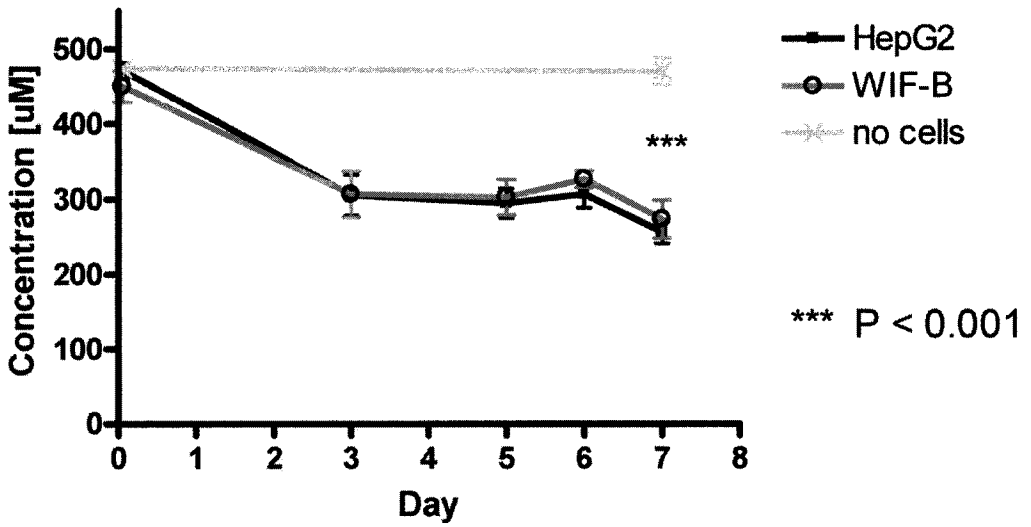

Figure 5.8 Disappearance of DEHA in samples of HepG2 and WIF-B cells with 500μM DEHA added at day 0 (n=3, bars indicate standard deviation)

Figure 5.8 showed that the rate of DEHA metabolism in WIF-B and HepG2 Cells is statistically similar. There was no significant difference between the data points for the cell lines according to the Bonferroni posttests. This was confirmed, using a two-way anova which showed that the cell type was not significant; however, the day was. Gas chromatography showed that the level of DEHA was reduced by 150μM in the system. Control samples, without cells, were also analyzed. These showed no reduction in DEHA concentration and no metabolites were found.

Figure 5.8 demonstrated that the first hydrolysis step in the breakdown of di(2-ethylhexyl) adipate, into 2-ethylhexanol and mono(2-ethylhexyl) adipate, happened at the same rate for both cell lines. As a result, this allows for the comparison of metabolite formation without having to compensate for different rates of plasticizer degradation.

In Figure 5.8, it appears that most of the degradation takes place by day 3. There was no significant difference between the samples at day 3 and day 7, for either cell line. It was hypothesized that the reason the cells were not able to further reduce the DEHA concentration was either that the degradation process was inhibited by the resulting production of 2-ethylhexanol or that the presence of this alcohol was having a toxic effect on the cells. To test this, the cells were incubated in vials for 3 days in the culture media and then at day 3, DEHA was added. As seen in Figure 5.9, there was no degradation of DEHA when it was added at day 3 and there was no statistical difference between the two cell lines.

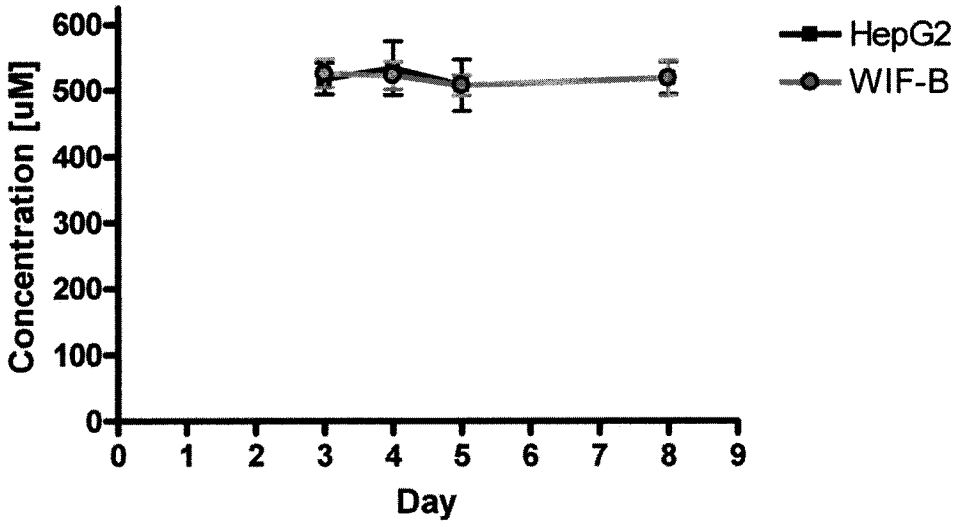

Figure 5.9 Disappearance of DEHA in samples of HepG2 and WIF-B cells with 500μM DEHA added at day 3 (n=3, bars indicate standard deviation)

5.4 Monitoring 2-Ethylhexanol

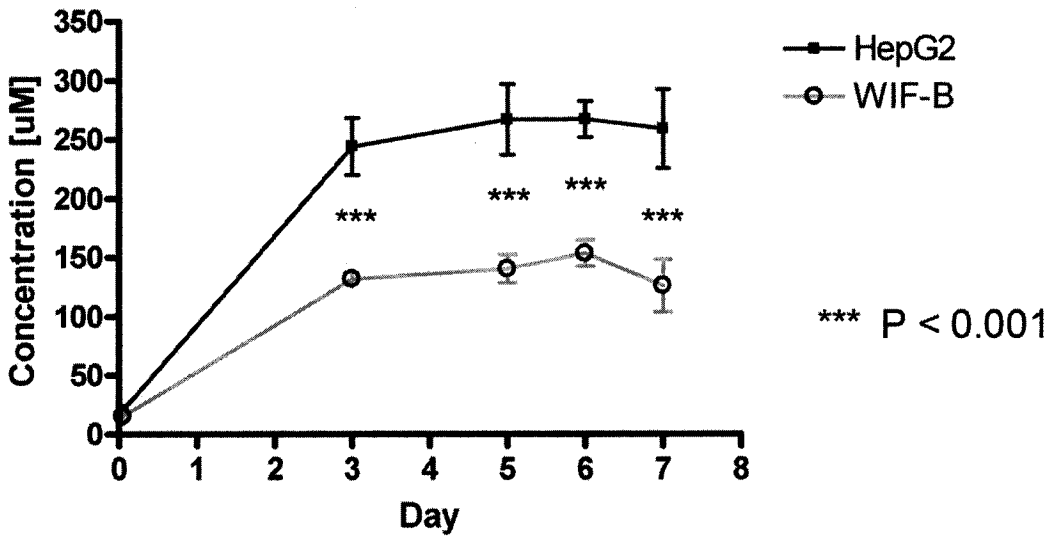

Figure 5.10 Appearance of 2-ethylhexanol in samples of HepG2 and WIF-B cells with 500 μM DEHA (n=3, bars indicate standard deviation)

As the concentration of DEHA decreases in the system, it is expected that the concentration of 2-ethylhexanol will increase, as it is hypothesized to be a metabolite. Figure 5.10 shows the appearance of 2-ethylhexanol in both cell systems. This formation is evidence of DEHA metabolism in both cell lines.

In both systems, the alcohol concentration appears to reach a constant level after 3 days. The concentration of alcohol in the HepG2 samples was approximately 250μM and in the WIF-B samples, was approximately 130μM. This constant level of 2-ethylhexanol obtained from day 3 to day 7, corresponds to the constant level of DEHA that was observed at the same time.

A two-way anova showed that both the cell type and day were significant as well as there being some interaction of these variables. The amount of 2-ethylhexanol was much lower in the WIF-B than in the HepG2 samples. This was shown to be significant by Bonferroni posttests, where $P< 0.001$. The lower concentration of 2-ethylhexanol in the WIF-B cell samples reflects their ability to metabolize the alcohol with more efficiency than HepG2 cells, as a result of having higher levels of active alcohol dehydrogenase. In the HepG2 samples, there is a higher concentration of 2-ethylhexanol in the HepG2 samples as the alcohol tends to build up.

In order to confirm the difference in the degradation of 2-ethylhexanol by the two cell lines, an additional experiment was performed. The cells were seeded

at a density of $1x10^6$ cells/mL and 2-ethylhexanol was added at a concentration of 200μM, in 0.5% v/v DMSO. This was similar to the amount of 2-ethylhexanol that was observed in the previous experiment, as a result of DEHA degradation.

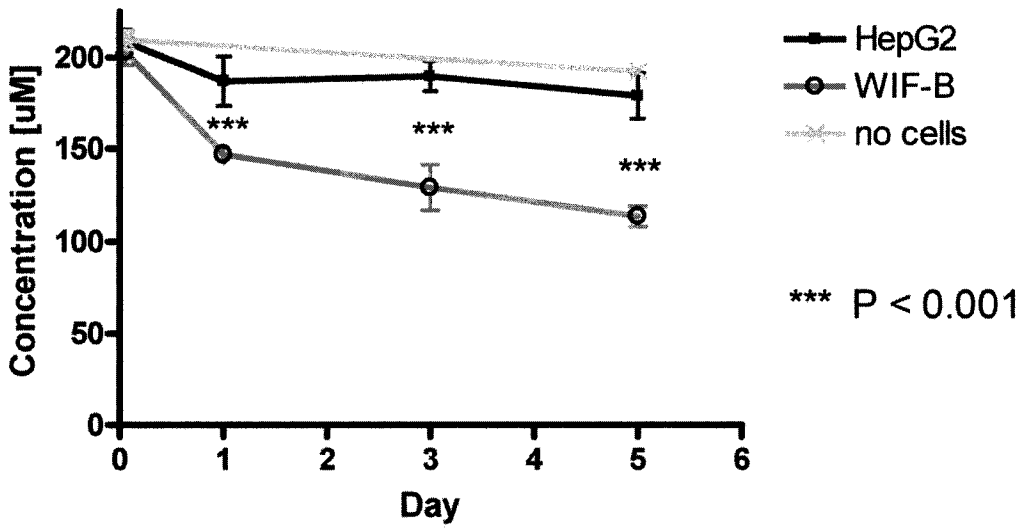

Figure 5.11 Disappearance of 2-ethylhexanol in samples of HepG2 and WIF-B cells with 200μM 2-ethylhexanol (n=3, bars indicate standard deviation)

A two-way anova analysis of the data in Figure 5.11 showed that the cell type and the day were significant and there was some interaction of these two variables. As shown in Figure 5.11, no significant degradation of the alcohol was observed with HepG2 cells. After 5 days, the level of alcohol was statistically similar to the control samples, which did not have cells. The controls showed negligible loss of 2-ethylhexanol, which demonstrates minimal evaporation. The WIF-B cells were able to reduce the amount of 2-ethylhexanol present. This increased alcohol metabolism is evident after only 1 day. There is a sharp reduction in alcohol concentration after the first day and between day 1 and day 5

there is a linear decrease in alcohol, (R^2 value 0.9989). The difference between the two cells lines was highly significant at all data points, ($P < 0.001$, Bonferroni post tests). This serves to validate that the observed degradation is enzyme regulated and not physical degradation.

5.5 2-Ethylhexanoic Acid Formation

As 2-ethylhexanol is broken down, it was expected that further metabolites, like 2-ethylhexanoic acid, would be observed.

Day	HepG2 [μM]	WIF-B [μM]
0	0 ± 0	0 ± 0
3	0 ± 0	2.67 ± 0.14
5	0 ± 0	3.56 ± 0.08

Table 5.1 2-ethylhexanoic acid appearance in HepG2 and WIF-B cells

After adding either DEHA or 2-ethylhexanol to WIF-B cells, the gas chromatographs revealed small amounts of the acid at day 3 and day 5. However, no acid was detected in the HepG2 samples. This is expected, as HepG2 cells should have limited alcohol metabolism (Figure 5.11).

6 Discussion

In this work, it has been demonstrated that DEHA can be partially degraded by HepG2 or WIF-B cells. There have been a number of publications showing that this compound can be degraded by microbial cells[1, 2, 9, 10, 12] and now, these results show that mammalian cells can metabolize DEHA. The degradation pathway was similar to bacterial degradation, as the metabolites observed are those that would be predicted from previous work on bacteria and yeast[2]. However there were significant differences in the rate and degree of degradation between the bacterial and mammalian cell lines as well as between the mammalian cell lines.

Neither mammalian cell line generated measurable amounts of mono-2-ethylhexyl adipate or 2-ethylhexanal. This is similar to the earlier experiments with microbial systems[2]. This work suggests that the biodegradation of either phthalate or adipate diesters of 2-ethyl hexanol do not generate measurable amounts of monoester metabolites, unless appreciable amounts of surfactant are present[40]. This has yet to be proven in mammalian systems.

The other two predicted metabolites not detected in the mammalian experiments were 2-ethylhexanal and adipic acid (Figure 6.1). These metabolites were likely produced but not beyond our detection limits. The microbial studies showed that 2-ethylhexanal was volatile and only observed in the gas phase leaving the bioreactor. Gases lost from the mammalian cell lines were not

collected. Any adipic acid released from the degradation would have been either easily physically degraded or found in the aqueous phase which was not analyzed.

Combined, these results show that the mammalian cell lines have the same ability to degrade the adipate plasticizer similar to bacteria and yeast investigated earlier. Furthermore, all of the evidence is consistent with the same mechanism of degradation starting with hydrolysis of the ester bonds followed by oxidation of the released alcohol.

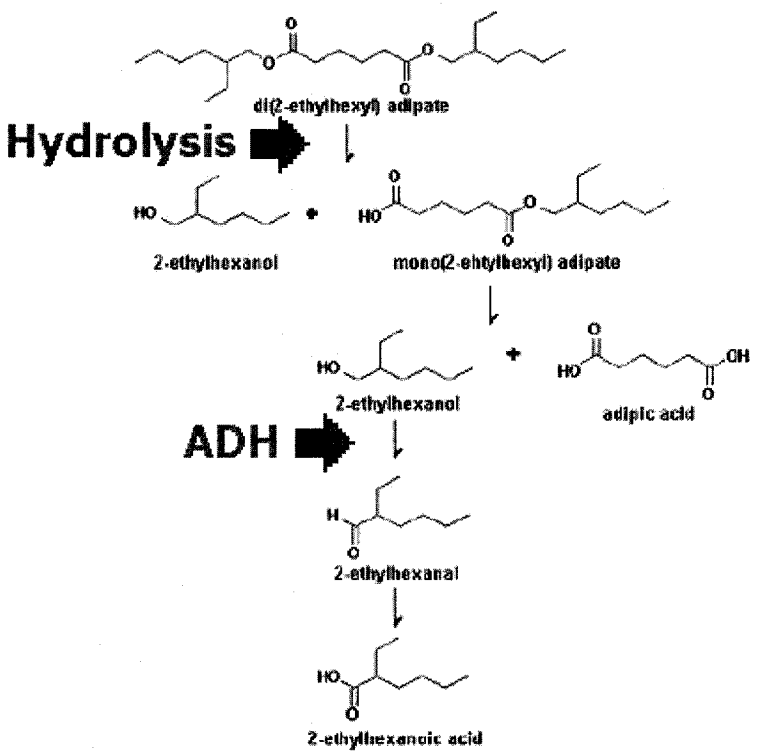

Figure 6.1 DEHA degradation in bacteria, fungi and yeast (30).

The steps of the degradation are expected to be mediated by enzymes in both the microbes and the mammalian cells. The results presented here support this conclusion and demonstrate that the degradation is truly biologically mediated. This can be seen in the differences in alcohol degradation of the two cell lines. WIF-B cells have been shown to have increased active alcohol dehydrogenase enzymes[32]. This allows them to metabolize alcohol much better then HepG2 cells, which tend to lose their hepatocyte specific functions in culture[32]. The result is that as the alcohol is released by hydrolysis of the DEHA, it cannot be degraded by the HepG2 cells and merely increases in concentration as long as the adipate ester bonds are being hydrolyzed. However, the WIF-B cells do have active alcohol dehydrogenase and, as a result, the level of 2-ethylhexanol is much higher in HepG2 samples than it is in WIF-B samples.

No further degradation of DEHA was observed after 3 days. It was hypothesized that the reason the cells were not able to further reduce the DEHA concentration was either that the degradation process was inhibited by the resulting production of 2-ethylhexanol (Figure 5.10) or that the presence of this alcohol was having a toxic effect on the cells (Figure 5.6, Figure 5.7). These scenarios were shown not to be the case. In an additional experiment, DEHA was only added at day 3 to avoid a buildup of 2-ethylhexanol in the system by day 3. Figure 5.9 shows that if DEHA is added day 3, after the cells have been in sealed vials in the incubator for 3 days, they were no longer capable of degrading DEHA. Therefore, the cells' inability to reduce the amount of DEHA in the

system was likely a result of the reduced viability or loss of function of the cells at this point in time. The media in the vials was yellow, not reddish/pink, indicating that the pH was no longer neutral. This could be a result of the fact that the vials were capped and this limits the gas exchange between the samples and the surrounding 5% CO_2 atmosphere, which buffers the pH of the media.

When DEHA is hydrolysed, 2-ethylhexanol is produced, as shown in Figure 5.10. Given that the rate of DEHA hydrolysis has been observed to be similar for the two cell lines (**Figure 5.8**). The fact that the amount of alcohol is different points to a cell specific metabolism and therefore biodegradation. There was a much smaller amount of alcohol observed in the WIF-B samples. This can be attributed to the fact that WIF-B cells have been transfected in order to express much more alcohol dehydrogenase than HepG2 cells. They have approximately twice the alcohol dehydrogenase of freshly isolated hepatocytes[32] and in culture, freshly isolated hepatocytes lose alcohol dehydrogenase expression. Therefore, WIF-B cells were able to metabolize the alcohol much more effectively then HepG2 cells and it does not accumulate in the system to the same level.

These conclusions can be further support by considering the appearance of 2-ethylhexanoic acid. Given that alcohols are regularly metabolized by mammalian liver cells, it is anticipated that 2-ethylhexanol will also be degraded. Therefore, it is expected that a further metabolite, such as the 2-ethylhexanoic acid observed in bacterial biodegradation (Figure 6.1), will be found. There was

evidence of 2-ethylhexanoic acid production in the WIF-B samples and this was not seen in the HepG2 samples. This confirmed that alcohol dehydrogenase, found in the WIF-B cells, was necessary to metabolise 2-ethylhexanol. None of the many experiments with HepG2 cells ever produced any measurable amounts of the acid.

Considering the limitations of HepG2 cells as a model of mammalian liver cells, it was expected that only WIF-B cells would be able to degrade pure 2-ethylhexanol and this was seen to be the case. When 2-ethylhexanol was the starting compound, there was an obvious difference in the degradation by the two cell lines, as seen in Figure 5.6. HepG2 cells were not able to significantly degrade 2-ethylhexanol. However, WIF-B cells degrade the alcohol rapidly during the first 24 hrs of exposure and continue to degrade it at a constant rate until day 5, when the experiment ends. This decrease in the rate of degradation was likely due to the reduced viability of the cells after 3 days as demonstrated in Figure 5.9.

All of these results show that alcohol dehydrogenase is integral to the degradation of DEHA by mammalian cells, by oxidizing the alcohol. Biodegradation by WIF-B cells is responsible for the observed decrease in 2-ethylhexanol. This is not physical degradation, as that would take place in the HepG2 samples also. As WIF-B cells are a more accurate model of liver cells,

DEHA cannot be assumed to be inert in mammals; it can be broken down and metabolites will appear.

An important consideration of this observed degradation of the adipate plasticizer is the different possible mixtures of metabolites and their toxicity. DEHA exhibited a toxic effect on both cell lines, as demonstrated by the MTT assay. This could be a direct result of the DEHA itself or perhaps the metabolites were exerting an adverse effect on the health of the cells. The toxicity was more pronounced in the HepG2 samples then the WIF-B samples, though this was not statistically significant.

The toxicity of alcohols on mammalian cells has been well demonstrated, though mainly with ethanol. In mammals, alcohol abuse results in fatty liver, hepatocyte necrosis fibrosis and ultimately cirrhosis[32]. In the HepG2 samples, 2-ethylhexanol tends to build up, as HepG2 cells have minimal alcohol dehydrogenase. However, the WIF-B cells do only marginally better. This could be attributed to ability of the WIF-B cells to metabolize 2-ethylhexanol better than HepG2 cells. As a result, the alcohol does not accumulate in the system to the same extent.

It is difficult to investigate the toxicity of plasticizers and their metabolites *in vivo*. The majority of studies are performed on the most common plasticizer, DEHP. Unlike these *in vitro* experiments, the one of the most common

metabolites observed *in vivo* is the monoester. In monkeys and man, DEHP is excreted in urine, as conjugated (glucuronide) oxidation products of mono(2-ethylhexyl) phthalate[22, 41]. In addition, the concentration of metabolites found depends on the species, with significant differences being observed between rats and men[17]. The presence of the monoester shows plasticizers are broken down *in vivo* and there is the potential for metabolite accumulation. *In vitro* experiments allow for a simplified model of plasticizer interaction and easier measurement of small metabolites. These experiments are important to understanding the effects of plasticizers on specific cells in a more controllable environment.

6.1 Future work

There are many future experiments that can be done to improve the understanding of the results presented here. Firstly, since DEHA degradation appears to stop at day 3 in capped vials, experiments should be done to test the hypothesis that this is a result of limited gas exchange and consequently, reduced viability of the cells. To do this, degradation experiments will be performed with the cap off the vials in order to allow the cells adequate gas exchange, though this leads to the potential loss of volatile metabolites. Secondly, the vapour phase of the samples should be analyzed. This can be done using headspace gas chromatography. It is possible that 2-ethylhexanal and some evaporated 2-ethylhexanol may be found in these samples. These experiments can also be repeated with additional plasticizers, in particular the most common plasticizer, DEHP. These experiments should be performed using WIF-B cells, as they

provide a better model for human hepatocytes than HepG2 cells. The metabolites observed in mammalian degradation can then be compared with ones occurring due to degradation by bacteria. In addition, the effect of plasticizers on hepatocytes and other mammalian cells can be studied further by examining morphological changes, gene expression and using RNA/protein changes. This will provide insight into the consequences of plasticizer exposure on a cellular level.

7 Conclusion

The experiments described in this thesis have shown that DEHA exposure results in noticeable toxicity to both HepG2 and WIF-B cell lines. It has been shown that like bacterial cells, mammalian cells can metabolize DEHA and produce similar metabolites. This strongly supports the hypothesis that the degradation pathway of DEHA in mammalian cells is the same as in bacteria, fungi and yeast.

In both cell lines DEHA was hydrolysed to produce measurable amounts of 2-ethylhexanol. It was observed that HepG2 cells were not able to metabolize the intermediate 2-ethylhexanol because of their limited alcohol dehydrogenase activity. However, WIF-B cells were able to reduce the amount of 2-ethylhexanol in the system and produce a further metabolite, 2-ethylhexanoic acid. This proved that alcohol dehydrogenase is essential to the breakdown of 2-ethylhexanol and therefore, DEHA is biodegraded and not physically degraded beyond the alcohol.

In these experiments, the degradation took place by day 3. It is suspected that by this time, the cells have limited viability after being in vials with limited gas exchange for 3 days. Further experiments will be done with the caps off, to test this hypothesis. The results of these degradation experiments also support the idea that WIF-B cells are a better model of human liver cells than HepG2 cells and therefore this cell line should be used in future experiments.

Most importantly, this work has shown that DEHA cannot be assumed to be inert in mammalian systems. Metabolites are formed and these metabolites are known to be more toxic than the initial plasticizer. This toxicity should be considered, especially for people or patients with a high level of exposure to plasticizers.

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Appendix A: Chemical List

Chemical	Purity	Supplier	
2-Ethyl hexanal	99%	Sigma Aldrich, Montreal, QC	
2-Ethyl hexanoic Acid	99%	Sigma Aldrich, Montreal, QC	
2-Ethyl hexanol	99%	Sigma Aldrich, Montreal, QC	*
Chloroform	99.9%	Fisher Scientific, Montreal, QC	
Di(2-ethylhexyl) adipate	99%	Sigma Aldrich, Montreal, QC	*
Dimethyl sulfoxide	99%	Fisher Scientific, Montreal, QC	*
Ethanol	85%	Anachemia Science, Montreal, QC	
Pentadecane	99%	A&C American Chemicals, Montreal, QC	
Sulfuric Acid	99%	Anachemia Science, Montreal, QC	

*denotes chemicals sterilized by filtration

Appendix B: Media Specifications

Eagle's Minimum Essential Medium (EMEM) Formulation

Catalog No. 30-2003

Inorganic Salts (g/liter)

$CaCl_2$ (anhydrous)	0.20000
$MgSO_4$ (anhydrous)	0.09767
KCl	0.40000
$NaHCO_3$	1.50000
NaCl	6.80000
$NaH_2PO_4 \cdot H_2O$	0.14000

Amino Acids (g/liter)

L-Alanine	0.00890
L-Arginine·HCl	0.12640
L-Asparagine·H_2O	0.01500
L-Aspartic Acid	0.01330
L-Cystine·2HCl	0.03120
L-Glutamic Acid	0.01470
L-Glutamine	0.29200
Glycine	0.00750
L-Histidine·HCl·H_2O	0.04190
L-Isoleucine	0.05250
L-Leucine	0.05250
L-Lysine·HCl	0.07250
L-Methionine	0.01500
L-Phenylalanine	0.03250
L-Proline	0.01150
L-Serine	0.01050
L-Threonine	0.04760
L-Tryptophan	0.01000
L-Tyrosine·2Na·2H_2O	0.05190
L-Valine	0.04680

Vitamins (g/liter)

Choline Chloride	0.00100
Folic Acid	0.00100
myo-Inositol	0.00200
Nicotinamide	0.00100
D-Pantothenic Acid (hemicalcium)	0.00100
Pyridoxine·HCl	0.00100
Riboflavin	0.00010
Thiamine·HCl	0.00100

Other (g/liter)

D-Glucose	1.00000
Phenol Red, Sodium Salt	0.01000
Sodium Pyruvate	0.11000

Phone 800-638-6597
703-365-2700
E-mail: news@atcc.org
Web site: www.atcc.org

F-12K Medium

Kaighn's Modification of Ham's F-12 Medium

Formulation

Catalog No. 30-2004

Inorganic Salts (g/liter)

$CaCl_2 \cdot 2H_2O$	0.13524
$CuSO_4 \cdot 5H_2O$	0.0000025
$FeSO_4 \cdot 7H_2O$	0.000834
$MgCl_2 \cdot 6H_2O$	0.10572
$MgSO_4$ (anhydrous)	0.19264
KCl	0.28329
KH_2PO_4 (anhydrous)	0.05852
$NaHCO_3$	1.50000
Na_2HPO_4 (anhydrous)	0.11502
NaCl	7.59720
$ZnSO_4 \cdot 7H_2O$	0.000144

Amino Acids (g/liter)

L-Arginine (free base)	0.42140
L-Alanine	0.01782
L-Asparagine·H_2O	0.03020
L-Aspartic Acid	0.02662
L-Cysteine·HCl·H_2O	0.07024
L-Glutamic Acid	0.02942
L-Glutamine	0.29220
Glycine	0.01501
L-Histidine·HCl·H_2O	0.04192
L-Isoleucine	0.00782
L-Leucine	0.02624
L-Lysine·HCl	0.07304
L-Methionine	0.00895
L-Phenylalanine	0.00991
L-Proline	0.06906
L-Serine	0.02102
L-Threonine	0.02382
L-Tryptophan	0.00408
L-Tyrosine (free base)	0.01087
L-Valine	0.02342

Vitamins (g/liter)

D-Biotin	0.0000733
Choline Chloride	0.01396
Folic Acid	0.00132
Hypoxanthine	0.00408
myo-Inositol	0.01802
Nicotinamide	0.0000366
D-Pantothenic Acid (hemicalcium)	0.000477
Putrescine·2HCl	0.000322
Pyridoxine·HCl	0.0000617
Riboflavin	0.0000376
Thiamine·HCl	0.000337
Thymidine	0.000727
Vitamin B-12	0.001355

Other (g/liter)

D-Glucose	1.26000
Phenol Red, Sodium Salt	0.00332
Sodium Pyruvate	0.22000
Lipoic Acid	0.00021

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C. 12500X Linoleic Acid (1X = 0.08mg/L or 0.04 mg/500ml)

Materials:

Linoleic acid minimum 99%, sigma L1376 100mg

Protocol:
1. To 100 mg ampule, add 1 ml 95% ethanol = 100 mg/ml,
2. Dilute 1:100 in 95% ethanol for 1mg/ml stock.
3. Store – 20 C tightly sealed

I. 1000x HAT Stock

Materials:

- Hypoxanthine, Anhydrous M.W. 136.1, Sigma Cat. #H-9636
- Aminopterin, Anhydrous M.W. 440.4, Sigma Cat. #A-3411
- Thymidine, Anhydrous M.W. 242.2, Sigma Cat. #T-1895

Protocol:

1. Add to 136.12 mg Hypoxanthine 0.6 ml 1 N NaOH and dissolve.
2. Add 1.76 mg Aminopterin to a couple drops of 1 N NaOH and dissolve.
3. Combine #1, #2 and 38.76 mg Thymidine with 100 ml with DDH_2O.
4. Sterile filter (0.22 □ m).
5. Make 5ml aliquots, incubate overnight at 4° C and then store at -20° C.

II. 500x Amphotericin B (Fungizone) Stock (250 m g/ml)

1. Amphotericin B (Fungizone), Sigma Cat. #A-2942.