

**AN *IN VITRO* METHOD FOR OBTAINING
BIOTRANSFORMATION RATES FOR BENZO[A]PYRENE
AND DIETHYL PHTHALATE USING THE SPRAGUE-
DAWLEY RAT SMALL INTESTINE**

by

Stephanie Ko

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APPROVAL

Name: Stephanie Ko
Degree: Masters of Resource Management
Project No.: 509
Title of Thesis: An *in vitro* method for obtaining biotransformation rates for benzo[a]pyrene and diethyl phthalate using the Sprague-Dawley rat small intestine

Examining Committee:

Chair: **Juan Jose Alava**
PhD candidate
School of Resource and Environmental Management,
SFU

Dr. Frank A.C.P. Gobas, Professor
Senior Supervisor
School of Resource and Environmental Management,
SFU

Dr. Margo M. Moore, Professor
Supervisor Committee Member
Department of Biological Sciences, SFU

Date Defended/Approved: December 10, 2010



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ABSTRACT

An *in vitro* method for measuring depletion rates for two hydrophobic chemicals, benzo[a]pyrene and diethyl phthalate, has been developed using enterocytes from the proximal 50 cm of the rat small intestine.

CYP1A1 activity of the enterocytes was determined and compared to the activity in rat liver S9 fractions by measuring luminescence. The CYP1A1 activity in S9 fractions was approximately 28 times greater than enterocytes (normalized to 10^6 cells).

The apparent depletion rate of benzo[a]pyrene was found to be $-0.65(\pm 0.31 \text{ S.E.}) \times 10^{-3} / \text{min} / 10^6_{\text{intestinal cells}}$ (n=1) whereas the depletion rate for diethyl phthalate was $-0.049(\pm 0.11 \text{ S.E.}) / \text{min} / 10^6_{\text{intestinal cells}}$ (n=3, $p < 0.05$).

Using isolated rat intestinal cells may not be the best method for measuring depletion rates in the small intestine because of the low CYP1A1 enzymatic activity in the cells, the labour-intensive procedure for isolating the cells, and the presence of mucous in the cells.

Keywords: Benzo[a]pyrene; Diethyl phthalate; *in vitro*; rat intestine; enterocytes; Cytochrome P450 1A1 activity.

DEDICATION

I dedicate this work to my parents who have whole-heartedly supported me in my pursuit of higher education. Without them, I would not be the person I am today nor will I be writing this dedication section in the first place!

I would also like to dedicate this work to all my teachers throughout my elementary, secondary and post-secondary school years for inspiring me to attain greater knowledge and for providing me the opportunity to do so.

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GLOSSARY

ACN	Acetonitrile
B	Bioaccumulation
BaP	Benzo[a]pyrene
BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
CAS no.	Chemical abstracts service number
CEPA	Canadian Environmental Protection Act
CMP	Chemicals Management Plan
C_{fish}	Concentration of a compound in fish
C_{octanol}	Concentration of a compound in octanol
C_{water}	Concentration of a compound in water
CYP450	Cytochrome P450 enzymes
d	Day(s)
DEHP	Di-(2-ethylhexyl)-phthalate
DEP	Diethyl phthalate
df	Degrees of freedom
DSL	Domestic Substances List
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
G6P	Glucose-6 phosphate
G6PD	D-glucose-6-phosphate (disodium salt hydrate)
GC-MS	Gas chromatography-mass spectrometer
GIT	Gastrointestinal tract
GST	Glutathione
h	Hour(s)
H-bonding	Hydrogen bonding
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICp	Inhibiting concentration for a (specified) percentage of the population to cause an effect
ICp ₅₀	Inhibiting concentration for 50% of the population
I.S.	Internal standard
iT	Inherent toxicity
k_m	Biotransformation rate
LC ₅₀	Lethal concentration (of a compound) to cause death for 50% of the population
Log K _{ow}	Log octanol-water partition coefficient
LRP	Lipoprotein receptor-related proteins

MEM	Minimum Essential Medium Eagle
MEP	Monoethyl phthalate
MRP1	Multidrug resistance-associated protein family
m/z	Mass-to-charge ratio
NADP	B-nicotinamide adenine dinucleotide phosphate
NADPH-GS	Nicotinamide adenine dinucleotide phosphate-generating system
NOEC	No observed effect concentration
P	Persistence
PBDE	Polybrominated diethers
PBT	Persistence, bioaccumulative, toxic
PCBs	Polychlorinated biphenyls
PEs	Phthalate esters
PAHs	Polycyclic aromatic hydrocarbons
P-gp	P-glycoprotein
PMSF	Phenylmethylsulfonyl fluoride
POPs	Persistent organic compounds
pST	Phenol sulfotransferase
PVC	Polyvinyl chloride
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation and Authorization of Chemicals
S9	Supernatant after centrifuging at 9000g
S.D.	Standard deviation
S.E.	Standard error
SOCs	Semivolatile organic compounds
T	Time or Toxic
$t_{1/2}$	Half-life
TSCA	Toxic Substances Control Act
UNEP	Uridine diphosphate
UDP	United Nations Environmental Programme
US EPA	United States Environmental Protection Agency

1: INTRODUCTION

1.1 Background

Thousands of synthetic chemicals such as PAHs, PCBs, pesticides, polybrominated diphenyl ethers (PBDEs), phthalates, and others have been in commerce and eventually released into the environment since the turn of the century. Some of these synthetic chemicals have been shown to cause harm to the environment and human life (Landrigan et al., 2002; Toppari et al., 1996; Guillette and Guillette, 1996). Currently, we are only beginning to understand the adverse effects these chemicals will cause us, and our children. If we are to ensure that future generations can enjoy a healthy life then we must take action to ensure that the harmful chemicals are phased out of production and use.

To protect the environment and human health, government agencies around the world have taken steps towards identifying the chemicals that are most harmful to human health and the environment. One of the global treaties with regards to chemical regulation, is the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention), which was signed by over 90 nations (including Canada, the United States and the European Union) in May 2001 and which came into force in May 2004 (Lallas, 2001). The Stockholm Convention is a global treaty designed to protect human health and the environment from persistent organic pollutants (POPs) by elimination or severely restricting the production and use of several POP pesticides and industrial chemicals. Initially,

12 POPs were identified to be phased out of production immediately with another nine added to the list in 2009 (Appendix A).

The Stockholm Convention requires that Parties take measures to “eliminate or reduce the release of POPs into the environment” (UNEP, 2001) and several national and international chemical management programs such as the Canada’s “Canadian Environmental Protection Act” (CEPA) (Government of Canada, 2006), the European Union’s (EU) “Registration, Evaluation, Authorization and Restriction of Chemicals” (REACH) program (Commission of the European Communities, 2007) and the United States’ “Toxic Substances Control Act” (TSCA) (US EPA, 1976) aim to do just that.

To identify the chemicals that are most harmful, agencies rely on three characteristics of the chemical – persistence, bioaccumulation and inherent toxicity, which together comprise of the “PBT” characteristics. The aim of classifying chemicals in terms of their PBT characteristics is to identify chemicals that may present a risk to humans and the environment.

In Canada, the Canadian Environmental Protection Act (CEPA) requires that the government assess and categorize approximately 23,000 substances in the Domestic Substances List (DSL) according to their PBT characteristics by September 2006 (CEPA, 1999). The DSL consists of substances (<100 kg) that were manufactured or imported into Canada that were in Canadian commerce or used for commercial manufacturing purposes in Canada between January 1st, 1984 and December 31st, 1986 (CEPA, 1999). These POPs are chemical substances that remain unchanged in the environment for a long period of time

(persistent), that bioaccumulate in organisms (an increase in tissue concentration as they move up trophic levels), and that are toxic to the environment and human health (Lallas, 2001).

The process that Environment Canada follows to evaluate and screen substances on the DSL for PBT characteristics is shown in Figure 1. Once the data on a particular substance is collected, the data is evaluated for its potential to be persistence *or* bioaccumulative *and* inherently toxic to non-human organisms. If a substance is considered to be *probable* in being persistent in the environment (criteria outlined below) or bioaccumulate in organisms, and is inherently toxic to organisms, then that substance will undergo further evaluation.

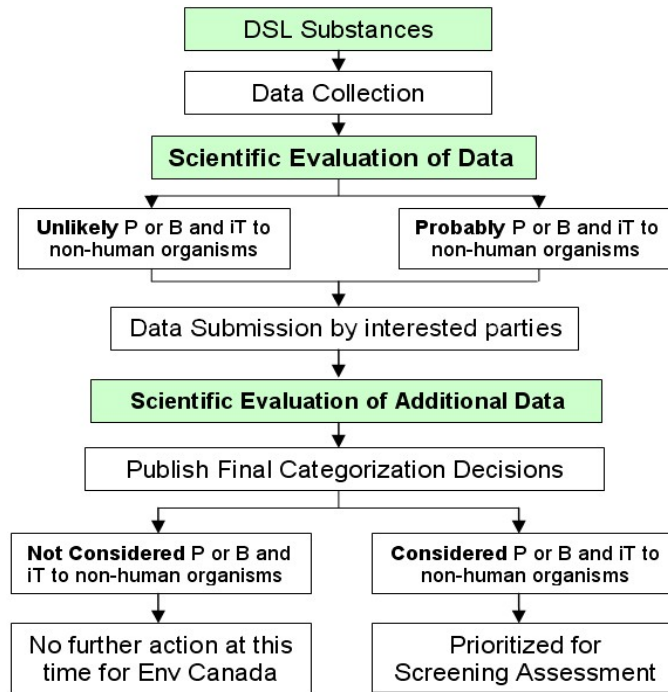


Figure 1. The categorization process Environment Canada uses for screening substances on the Domestic Substances List (DSL) (Environment Canada, 2006).

Chemicals that have been shown to be inherently toxic to humans or non-human organisms and are either persistent or bioaccumulate, or may present the greatest potential for exposure to Canadians will undergo a screening level risk assessment (Environment Canada, 2009b). After the chemical undergoes a screening level risk assessment, it is put into three categories: no further action, Priority Substances List or List of Toxic Substances (Schedule 1, possible Virtual Elimination). 'No Further Action' means that the substance deemed to be non-toxic or that it is toxic and action is taken to manage the risk. Chemicals on the Priority Substances List require further investigation into the environmental behavior of the chemical to determine whether they are toxic under CEPA. Substances on the List of Toxic Substances allow the government to proceed with implementing regulations, pollution prevention plans or environmental emergency plans to regulate the use and production of those substances (CEPA, 1999).

The categorization process was completed on September 14th, 2006 (Environment Canada, 2009a). Of the 23,000 chemical substances on the DSL, approximately 4000 substances require further attention and 500 are listed as High Priority Substances (Government of Canada, 2007b). High Priority chemicals are substances that meet the inherent toxicity criteria to humans and the greatest exposure potential for human exposure, or that meet the PBT characteristics to the environment (Government of Canada, 2007a).

In 2006, the Chemicals Management Plan (CMP) was introduced in Canada to manage chemical substances properly. There are three key elements to the CMP:

- challenge to industries and stakeholders
- other regulatory activities, and
- new investment in research and monitoring.

The challenge to industries and other stakeholders was developed to address 12 batches of high-priority chemicals for action, in groups of 12 - 20 (Government of Canada, 2007b). Approximately every three months, a new batch of chemical names are released for comment and information gathering. Industries and stakeholders are required to provide information that forms the basis for risk assessment, to identify industrial best practices in order to set the benchmarks for risk management and product stewardship, and to detail environmental release, exposure, and substance or product use. This information is used to assess risks to human and the environment, and to develop a course of action to address these risks (Government of Canada, 2007a).

In terms of regulatory action, some chemicals have been grouped according to their industrial sector (e.g. petroleum sector) so that a comprehensive risk assessment can be conducted.

The third key element to the CMP is to monitor chemicals in humans and the environment in order to identify and understand hazardous properties of high-risk chemicals, their fate in the environment, and how people and the

environment may be exposed and affected by these chemicals (Government of Canada, 2007a).

According to Environment Canada (2006), the criteria for categorizing chemicals according to their “PBT” characteristics are explained as follows: Persistence refers to the length of time a particular substance remains unchanged in the environment. It is measured by its half-life ($t_{1/2}$); the amount of time it takes for half of the initial quantity of the substance to degrade in a particular environmental medium. Persistence is a problem because the longer a substance remains unchanged in the environment, the higher the chance it has of being transported to the polar regions via the ‘Grasshopper Effect’ and exert its effects on more remote regions of the Earth (Semeena and Lammel, 2005). Some semivolatile organic compounds (SOCs) undergo multiple evaporation and condensation events that allow them to travel to the polar regions of the Earth, which is usually a long distances from where they were first deposited. Table 1 shows the persistence criteria that Environment Canada, the EU and the United States have set for substances in various media. The persistence criterion varies according to the type of medium the substance is present. The shortest persistence criterion is in air (2 days), the longest is in sediments (365 days) and the middle is in water (40 – 180 days; Table 1).

Table 1. An overview of regulatory persistence criteria of various agencies around the world.

Regulatory Agency	Persistence Criteria (half-life, $t_{1/2}$) (The substance fulfils the persistence criteria when:)		Program
	Medium	Criteria	
Environment Canada	Air	≥ 2 days	CEPA (1999)
	Water	≥ 182 days	
	Sediment	≥ 365 days	
	Soil	≥ 182 days	
European Union	Fresh-/estuarine water	> 40 days	REACH (2006)
	Fresh-/estuarine water (very persistent)	> 60 days	
	Marine water	> 60 days	
	Fresh-/estuarine water sediment	> 120 days	
	Fresh-/estuarine water sediment (very persistent)	> 180 days	
	Marine sediment	> 180 days	
	Soil	> 120 days	
US EPA	Water	> 60 days (SNUR)*	TSCA (1976)
	Water	> 180 days (BPT)**	

*SNUR = Significant New Use Rule: Exposure/release controls included in order; testing required.

**BPT = Ban Pending Testing: Deny commercialization; testing results may justify removing chemical from “high risk concern”

The second criterion that various national and international chemical regulatory agencies consider is the potential for a substance to bioaccumulate in aquatic organisms. Environment Canada (2006) defines it as “a process by which substances are accumulated in an organism directly from exposure to water and through consumption of food containing the substances”. There are several different ways of expressing the degree to which chemicals can bioaccumulate in organisms such as by the bioaccumulation factor (BAF) and bioconcentration factor (BCF). These BAF and BCF values may be estimated using the chemical’s $\log K_{ow}$ (octanol-water partition coefficient).

The BAF is the steady-state ratio of the concentration of a chemical in the organism (typically a fish) and the concentration of a chemical in water taking into consideration all routes of exposure (dermal surface, respiration, diet) and routes of elimination (respiration, fecal egestion, growth dilution or metabolism) (Environment Canada, 2006; Arnot and Gobas, 2006). The BAF is typically a field measurement. Equation 1 shows this ratio:

$$\text{BAF} = C_{\text{fish}}/C_{\text{water column}} \quad 1$$

Where C_{fish} (g/kg) is the concentration of the chemical in the fish, C_{water} (g/L) is the concentration of the chemical in the water and BAF is in L/kg.

The BCF is typically measured in the lab and takes into consideration all routes of exposure and elimination *except* dietary exposure. The BCF is expressed as (equation 2):

$$\text{BCF} = C_{\text{fish}}/C_{\text{water (lab)}} \quad 2$$

Where C_{fish} (g/kg) is the concentration of the chemical in the fish, C_{water} (g/L) is the concentration of the chemical in the water and BCF is in L/kg.

Lastly, the $\log K_{\text{ow}}$ refers to the ratio of the distribution of a chemical in octanol compared to that in water. The $\log K_{\text{ow}}$ can be expressed as (equation 3):

$$\text{Log } K_{ow} = \text{Log } (C_{\text{octanol}}/C_{\text{water}})$$

3

Where C_{octanol} (g/L) is the concentration of the chemical in octanol and C_{water} (g/L) is the concentration of the chemical in water; $\log K_{ow}$ is unitless as the units of C_{octanol} and C_{water} cancel out. The higher the value of BAF or BCF, the higher the propensity the chemical has to bioaccumulate in the organism. Similarly, the higher the $\log K_{ow}$ value is, the more lipophilic the chemical tends to be because more of it remains in octanol (a surrogate for lipids in organisms) compared to water (a polar solvent).

Table 2 shows an overview of the various BAF, BCF and $\log K_{ow}$ criterion values which agencies around the world use to assess the bioaccumulation potential of substances.

Table 2. An overview of regulatory bioaccumulation endpoints and criteria of various agencies around the world.

Regulatory Agency	Bioaccumulation Criteria (The substance fulfils the bioaccumulation criteria when:)			Program
	BAF (log values)	BCF (log values)	K_{ow} (log values)	
Environment Canada	$\geq 5\ 000$ (3.7)	$\geq 5\ 000$ (3.7)	$\geq 100\ 000$ (5)	CEPA (1999)
European Union 'bioaccumulative'	-	$\geq 2\ 000$ (3.3)	-	REACH (2006)
European Union 'very bioaccumulative'	-	$\geq 5\ 000$ (3.7)	-	REACH (2006)
US EPA (SNUR)	-	1 000-5 000 (3-3.7)	-	TSCA (1976)
US EPA (BPT)	-	$\geq 5\ 000$ (3.7)	-	US EPA. (2010)
UN Environment Programme	-	$\geq 5\ 000$ (3.7)	$\geq 100\ 000$ (5)	Stockholm Convention (2001)

Although the BAF is measured in the field and is therefore more reflective of how a chemical will likely behave in the natural environment, it is more difficult to obtain accurately compared to the BCF or $\log K_{ow}$ values because there are many variables affecting the BAF (such as climate, size of organism, food-web interactions, etc.). All these variables make it difficult to objectively compare one value to another (Arnot and Gobas, 2006). That may be the reason why only Environment Canada is using the BAF. The BCF, on the other hand, is measured in the lab under controlled settings and is easier to compare when variables are controlled.

Relative to BAF and BCF values, $\log K_{ow}$ values are easier to obtain. Basically, a certain amount of a chemical is deposited into a vial containing octanol and water. After some time, the concentration of the chemical in octanol and water is measured and K_{ow} is then calculated according to equation 3. Since K_{ow} values reflect passive chemical partitioning between a lipid surrogate (octanol) and water, it may oversimplify the many physiological processes organisms take up and eliminate the chemical especially when there are transport proteins involved.

The third criterion that is considered in a PBT evaluation is “inherent toxicity” (iT). Inherent toxicity refers to the “hazard a substance presents to an organism” (Environment Canada, 2006). Environment Canada prefers to use acute toxicity studies over chronic toxicity studies because there are more data available (shorter time duration means it is easier to obtain data) and QSAR (quantitative structure-activity relationship) models are available for acute

endpoints (Environment Canada, 2006). Table 3 highlights toxicity criteria from various regulatory agencies around the world. Table 3 shows that there is no commonality among the toxicity criteria of various agencies around the world.

Table 3. Toxicity criteria of various agencies around the world.

Regulatory Agency	Toxicity Criteria (The substance fulfils the toxicity criteria when:)		Program
	Exposure Duration	Criteria	
Environment Canada	Acute ⁺	LC ₅₀ (EC ₅₀)* ≤ 1 mg/L	CEPA (1999) (for aquatic species)
	Chronic ⁺⁺	NOEC** ≤ 0.1 mg/L	
EU	Chronic (marine or freshwater organisms)	NOEC ≤ 0.01 mg/L	REACH (2006)
	The substance is classified as carcinogenic (category 1 or 2), mutagenic (category 1 or 2) or toxic for reproduction (category 1, 2, or 3), or there is other evidence of chronic toxicity		
US EPA	“Develop toxicity data where necessary, based upon various factors, including concerns for P, B, other physical/chemical factors, and toxicity“.		US EPA (2010)

Acute⁺: for *Daphnia magna* – 48h LC₅₀; for rainbow trout (*Oncorhynchus mykiss*) – 96h LC₅₀

Chronic⁺⁺: for green algae (*P. subcapitata*) – 72h IC₅₀***; for larvae of freshwater midges (*Chironomus tentans* or *C. riparius*) – 10d LC₅₀; for larval fathead minnows (*Pimephales promelas*) 7d LC₅₀ or 7d ICp**** (Environment Canada, 2009).

*LC₅₀: the concentration of the substance in water causing death to 50% of the experimental organisms. EC₅₀ is the concentration of the substance in water inducing toxic effects on 50% of the experimental organisms.

** NOEC: Non-observed-effect concentration, where there is no observed adverse effect for the highest concentration of a substance in a toxicology study.

***IC₅₀: Inhibiting concentration for 50% of the population (e.g. decreased biomass)

****ICp: Inhibiting concentration for a (specified) percentage effect

To predict BCFs and BAFs for aquatic organisms, researchers often use log K_{ow} values and computer models. Computer models that are used to predict BCFs identify different ranges of log K_{ow} values that meet or exceed the criteria (Arnot and Gobas, 2006). Arnot and Gobas' paper (2006) report that, using a bioaccumulation criteria of 5000 L/kg, different computer models predicted different log K_{ow} ranges as having bioaccumulation potential. For example, Arnot and Gobas' paper states that the Mackay linear BCF model predicts a log K_{ow} value equal or greater than 5 that meets or exceeds the BCF criteria on a value of 5000 L/kg. The BCFWIN model (without any correction factors) predicts log K_{ow} values between 5.7 - 7.8 to exhibit a BCF criteria in excess of 5000 L/kg. Arnot and Gobas predicted log K_{ow} values between 4.9 - 8.8 for their own BCF model and 4.1 - 12.5 for their own BAF model to exceed the bioconcentration criteria of 5000, respectively. In addition, Arnot and Gobas assessed the probability of each model for generating Type II errors (false negative; predicting that a chemical does not meet the bioconcentration criteria when it actually does). These type II errors range from 5.6% (Arnot-Gobas BAF model) to 70.6% (BCFWIN model). This means that there can be much uncertainty when using computer models to generate BCFs from log K_{ow} values.

Arnot and Gobas (2006) showed that BAF values are greater than BCF values because of dietary uptake and magnification, trophic transfer, and sediment-water disequilibria, factors that are not present in the BCF. These factors show that BAF values are more reflective of the natural environment to which an organism is exposed and therefore, are more valuable when assessing

it against the bioaccumulation criteria of 5000. However, there just are not that many BAFs in literature. If there is a heavy reliance on using BCFs to access bioaccumulation criteria, then the probability of generating Type II errors regarding chemical regulations in terms of PBT criteria increases.

A way of including biological processes in terms of bioaccumulation potential may be to use an *in vitro* biotransformation system that can be used to extrapolate to whole body metabolism and refine BCF estimates since many more BCF data values are available compared to BAFs Cowan-Ellsberry (2008). Han and colleagues (2007) measured the depletion of the parent compound in trout liver S9 fraction and in primary hepatocytes isolated from the common carp. His results suggest that *in vitro* biotransformation data can be used to refine BCF model estimates. However, validations for a wider range of chemicals are needed. Cowan-Ellsberry et al. (2008) based their method on what pharmaceutical companies have been doing for years, which is to measure the route and fate of new drugs within the human body using *in vitro* studies.

Most pharmaceutical companies use the rat liver as a model system for studying the fate of new drugs in the human body. The liver is often the major site of metabolism in the body but the small intestine can also play a significant role in the biotransformation of chemicals in organisms (Doherty and Charman, 2002; Kaminsky and Zhang, 2003; Back and Rogers, 1987). Pharmacological research has shown that the first pass metabolism in the small intestine can affect the bioavailability of drugs before they reach the systemic circulation (Back and Rogers, 1987). There are many digestive enzymes such as proteases,

lipases, nucleases (Fearn and Hirst, 2006), transport proteins (Doherty and Charman, 2002) and CYP450 enzymes (Martignoni et al., 2006) in the intestine. Nutrients, drugs, and xenobiotics have to be absorbed by the intestinal cells (enterocytes), then travel through the portal vein to the liver where they are subject to biotransformation (Martignoni et al., 2006). Because of this relationship between the small intestine and liver, it is difficult to ascertain the role of the small intestine in terms of its contribution of overall biotransformation rate in organisms.

There are many studies that indicate the small intestine contributes substantially to overall first-pass metabolism of drugs such as cyclosporine, nifedipine, midazolam, verpamil and others (listed in Lin et al., 1999). Some studies have suggested that the contribution of the intestinal metabolism to overall first-pass effect is quite large (Wu et al., 1995; Zhang and Benet, 2001).

The goal of this project was to develop and evaluate a suitable *in vitro* method for measuring biotransformation rates of hydrophobic chemicals in rat intestinal cells. The two test chemicals were benzo[a]pyrene, a PAH and diethyl phthalate, a phthalate ester. The CYP1A1 activity in the rat small intestinal cells were measured and compared with rat liver S9 fractions to evaluate the use of isolated small intestinal cells in *in vitro* biotransformation rate studies.

1.2 Background on small intestinal metabolism

The primary function of the mammalian small intestine is to digest and absorb nutrients. The mammalian small intestine is divided into three sections. The slightly digested foodstuff passes from the stomach and into the duodenum

(proximal third of the small intestine), jejunum (middle third) and finally, into the ileum (distal third). The small intestinal morphology is particularly well-suited for digestion and absorption because of its extensive surface area resulting from the projection of many tiny villi (finger-like projections) (Kaminsky and Zhang, 2003).

Along the length of the small intestine in mammals, the villi are most populous in the duodenum and jejunum, and gradually decreases towards the ileum. The base of the villi are composed of undifferentiated cells, goblet cells, enteroendocrine cells and Paneth cells (Kaminsky and Fasco, 1992). These stem cells at the base of the crypt give rise to differentiated enterocytes (the villous epithelium) of which the tips contain carrier-mediated absorption proteins (Doherty and Charman, 2002), metabolizing enzymes such as the cytochrome P450 (CYP450) enzymes (Kaminsky and Fasco, 1992) and other proteins (Doherty and Charman, 2002). These CYP450 enzymes are responsible for metabolizing many xenobiotics, most notably the ubiquitous environmental contaminant, benzo[a]pyrene. The highest amount of these CYP450 enzymes are reported to be in the differentiated epithelial cells of the villus region and decline progressively towards the crypt region (van de Kerkhhof, 2007b) while UDP-glucuronyltransferase is more evenly distributed along the length of the villus (Borm et al., 1983). Hence, this gradient of enzymatically active cells near the proximal end of the small intestine and at the tips of the villi, while leading to a gradual decrease towards the distal end and the crypt cells, is crucial in understanding factors that affect the extent and rate of xenobiotic biotransformation in the small intestine of mammals.

In addition to containing digestive enzymes such as proteases, lipases, nucleases (Fearn and Hirst, 2006), and CYP450 enzymes, the small intestine contains transport proteins. These proteins, such as P-glycoprotein, Multidrug Resistance-Associated Protein Family (MRP), lung resistance protein (LRP) and other transporter proteins, are capable of recycling xenobiotic compounds between enterocytes and the lumen in rats and in humans (Martignoni et al., 2006; Doherty and Charman, 2002). This recycling increases the xenobiotic exposure to metabolizing enzymes and may decrease the chance of xenobiotic compounds reaching the systemic circulation.

Much of the research on *in vitro* BaP and phthalate biotransformation rates in rat intestines was performed in the 1960s-80s whereas most of the modern literature that focused on BaP biotransformation rates also included transport of metabolites (Table 4). In terms of studying phthalate diester biotransformation rates, only three articles were found pertaining to DEP whereas many more papers pertained to di-(2-ethylhexyl) phthalate (DEHP) biotransformation (Table 4).

Table 4. List of journal articles found that focused on BaP and DEP biotransformation rates in the small intestine of the rat

Journal Article	Subject	Model
Wattenberg et al., 1962	Benzpyrene hydroxylase activity in the gastrointestinal tract	Male SD-rats*; intestinal mucosa
Chhabra, 1974	A comparative study of xenobiotic-metabolizing enzymes in liver and intestine of various animal species	Male CDI strain of mice; microsomes
Stohs et al., 1977	Benzo[a]pyrene metabolism by isolated rat intestinal epithelial cells	Male SD-rats; enterocytes
Grafström, 1979	Xenobiotic metabolism by isolated rat small intestinal cells	Male SD-rats; enterocytes
Autrup et al., 1982	Comparison of benzo[a]pyrene metabolism in bronchus, esophagus, colon, and duodenum from the same individual	Male & female human intestinal tissue (age 18-65)
Salbe and Bjeldanes, 1985	The effects of dietary Brussels sprouts and <i>Schizandra chinensis</i> on the xenobiotic-metabolizing enzymes of the rat small intestine	Male SD-rats; <i>in vivo</i>
van de Wiel et al., 1992	Influence of long-term ethanol treatment on <i>in vitro</i> biotransformation of benzo[a]pyrene in microsomes of the liver, lung and small intestine from male and female rats	Male & female Wistar rats; microsomes
Rowland et al., 1977	Hydrolysis of phthalate esters by the gastro-intestinal contents of the rat (esters: dimethyl, diethyl, di- <i>n</i> -butyl, dicyclohexyl, di- <i>n</i> -octyl & di-(2-ethylhexyl) phthalate)	Male Wistar and SD-rats; small intestinal suspensions
Lake et al., 1977	The <i>in vitro</i> hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species (esters: diethyl, di- <i>n</i> -butyl, di- <i>n</i> -octyl, di-(2-ethylhexyl), dicyclohexyl phthalates; species: rat, baboon, ferret)	Male SD-rats; intestinal mucosal cell homogenates
White et al., 1980	Absorption and metabolism of three phthalate diesters by the rat small intestine (esters: dimethyl, di- <i>n</i> -butyl, di-(2-ethylhexyl) phthalates)	Male SD-rats; everted gut

*SD-rats – Sprague-Dawley rats

Many of the articles listed in Table 4 investigated the ability of the small intestine to metabolize BaP and DEP, *not* on their biotransformation rates. The three main papers that investigated the *in vitro* hydrolysis of DEP were performed in the late 1970's while most of the subsequent studies on phthalate ester metabolism were done using DEHP and other higher molecular weight phthalates.

Two study chemicals with different structures were chosen, a polycyclic aromatic hydrocarbon, benzo[a]pyrene and a phthalate ester, diethyl phthalate. Benzo[a]pyrene is one of the many polycyclic aromatic hydrocarbons (PAHs) that are ubiquitous environmental contaminants.

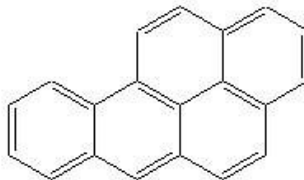


Figure 2. Structure of benzo[a]pyrene (BaP).

BaP (Figure 2) comes from a number of different sources such as from industrial processes, vehicle exhaust emissions, oil spills, cigarette smoke and cooked foods (Miller and Ramos, 2004). Of all the hundreds of PAHs that are detected in the environment, many of them, including BaP, has been recognized as carcinogenic. BaP is one of the 16 PAHs designated on the priority pollutant

list by US EPA (Cavret et al., 2004). Because BaP is a ubiquitous environmental contaminant and because it is converted to a carcinogen by metabolism, its metabolism has been extensively studied. BaP (CAS no. 50-32-8) has a log K_{ow} value of 5.98 and water solubility of 3.78 $\mu\text{g/L}$ (Hattermer-Frey and Travis, 1991). The high log K_{ow} value of BaP makes it a particularly suitable study chemical for this project because one of the goals of the experiment is to find biotransformation rates for hydrophobic compounds in the rat small intestine. The high log K_{ow} value for BaP yields a BCF value greater than 5000 from modelling; however, this overestimates its bioaccumulative potential because it is biotransformed into a more water-soluble compound and is excreted in the urine and feces (van Schooten et al., 1997).

Diethyl phthalate (DEP, CAS no: 84-66-2) is a lower MW phthalate with the following chemical structure in Figure 3:

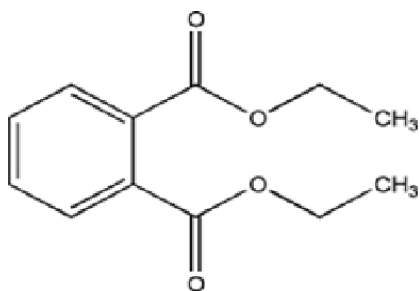


Figure 3. Structure of diethyl phthalate (DEP).

Phthalates, as a group of structures, are colourless liquids with a slightly aromatic odour and low volatility. Phthalate esters (PE) are divided into three general classes of use, depending on their molecular weights (MW). Lower-MW

PE (e.g. dimethyl and diethyl phthalates) are used as solvents in cosmetics (bath oils, eye shadow, perfumes, hairspray, nail polish, etc.) or as plasticizers of cellulose acetate polymers. Higher-MW PEs are used as solvents in some PVC products that are combined with other plasticizers in floor covers, or as a solvent or plasticizer in the cosmetic and pharmaceutical industries. The highest-MW PEs are used as plasticizers of PVC for wire and cable coverings, medical products and other consumer products (David and Gans, 2003). Studies have shown that short (acute) exposure to DEP is not a health concern (Sharpe and Irvine, 2004; Api, 2001), but because PEs are widely used and exposure to them is high, chronic toxicity is a concern because studies have shown that some phthalates (e.g. Di-*n*-butyl phthalate and di-(2-ethylhexyl) phthalate, DEHP; van Wezel et al., 2000; Latini et al., 2004)) are endocrine disruptors and may affect the reproductive tract of male rats which results in a decrease in sperm production (Mylchreest et al., 1999).

One of the most widely used PEs is di-(2-ethylhexyl) phthalate (DEHP) and much research has been done on this phthalate regarding its toxicity and human health effects. Comparatively, there are fewer studies on DEP, mainly because it is metabolized quickly (around 1-2 days) and likely will not bioaccumulate in the organism (Api, 2001). The impact of DEP on the organism will likely be less than DEHP because it is metabolized faster than DEHP and therefore, remains in the body for a shorter amount of time. A study conducted by Rowland and colleagues (1977) measured the metabolic rates of a few PEs in the small intestine of the rat and lighter MW PEs such as dimethyl phthalate

(DMP), DEP and dibutyl phthalate were metabolized more quickly than the heavier weight PEs, such as dicyclohexyl phthalate (DCP), DEHP and Di-*n*-octyl phthalate (DOP). In the body, DEP is believed to be hydrolyzed to its monoester derivative and the hydrolysis reaction is similar in rats and humans. The hydrolysis to the monoester can occur in the lumen of the GIT, in the intestinal mucosal cells, liver, kidney and lung (Api, 2001). The monoester derivative, monoethylphthalate, is fairly rapidly excreted in the urine with 47% of the dose excreted within 12 h, 82 % within 24 h and 90% within 48 h after oral administration to rats (Api, 2001).

Diethyl phthalate was chosen as the second test chemical because it is a PE, and is used in a wide variety of products such as plastic packaging films, cosmetic formulations (e.g. hairspray, nail polish, and perfumes), toiletries, medical treatment tubing, as an alcohol denaturant, a camphor substitute, wetting agent, dye application agent and a surface lubricant in food and pharmaceutical packaging (NTP, 2006). Diethyl phthalate is also metabolized quickly in the body (Api, 2001), it has a log K_{ow} value of 2.47 and it is soluble in water (1000 mg/L of water at 25°C) (Sabljic et al., 1995).

2: THEORY

2.1 *In vitro* methods for measuring biotransformation rates

There are many pharmaceutical studies that measure *in vivo* and *in vitro* xenobiotic intestinal metabolism because the peroral route is the most common mode of administering a drug (Kapp, 2007). Drugs that enter the body through the peroral route is subject to first-pass metabolism in the small intestine and liver. When measuring the extent of metabolism in the small intestine, researchers tend to focus on the type and amount of metabolites generated, or the amount of parent compound that is remaining after a certain time period. In most cases, the focus has not been on measuring biotransformation *rates*.

As noted above, the relationship between the small intestine and the liver in the body makes it difficult to separate the relative contributions of each organ to total body metabolism. Methods for studying intestinal metabolism (from whole organs to tissues to cells to sub-cellular fractions) include perfusion, tissue slices, cell cultures, isolated cells, and cell fractions (van de Kerkhof, et al., 2007a; Fix, 1996). For the sake of brevity, the focus of the following section will be a brief summary on subcellular fractions, S9 fractions, and microsomal fractions that are used for biotransformation studies because they are most similar to my chosen experimental method. For more information on different methods of *in vitro* biotransformation studies, please refer to van de Kerkhof et al. (2007a), Quaroni and Hochman (1996), Kaminsky and Fasco (1992) and Kimmich (1990).

2.1.1 Cellular fractions

Isolated enterocytes are often used in drug/xenobiotic metabolic studies. Their main advantage is that the extracellular environment can be highly controlled. Isolated enterocytes do not require the addition of add co-factors since these are intact cells and already contain their own co-factors. Isolated cellular fractions have been used extensively to study glucuronidation and sulphation of various xenobiotics (Bonkovsky, 1985). The disadvantages of using isolated cells are that the steps in preparing and isolating the cells are labour intensive and not easily standardized between laboratories. Moreover, studying drug transport is difficult because cell polarity is lost (Fearn and Hirst, 2006). In the small intestine, cells near the proximal end contain slightly different enzymes and in varying amounts compared to cells near the distal end (Kaminsky and Zhang, 2003). In addition, cells in the proximal end (villous tips) also vary in the type and amounts of enzymes contained within them compared to crypt cells (Kaminsky and Zhang, 2003). There are different ways to isolate intestinal epithelial cells. They include the vibrational method (Kaminsky and Fasco, 1992), the scraping method (Bonkovsky, 1985) and the elution method (Weiser, 1973).

2.1.1.1 Vibrational method

Kaminsky and Fasco (1992) describe a vibrational method where the small intestine is everted over steel rods and placed in a buffered solution containing ethylenediaminetetracetate (EDTA), and subjected to mechanical stress using a vibro-mixer. According to the degree of vibrational stress, either the villous or crypt cells become separated from the small intestine.

2.1.1.2 Scraping method

Scraping the mucosal side of the small intestine can also yield epithelial cells (Zheng et al., 1999) but Kaminsky and Fasco (1992) suggest that scraping action indiscriminately yields a mixture of villous tip, mid-villous and crypt cells while the action may damage the cells. In addition, this scraping action also dislodges cell clumps, cell debris, lamina propria, connective tissue and excessive amounts of mucous (Kimmich, 1990). This gelatinous mass creates cell clumps that prevent easy separation of the cells. Therefore, scraping of the intestine should only be used if the procedure does not require intact cells. However, the scraping method is the easiest and fastest method of obtaining cells compared to the vibrational or elution technique (Stohs et al., 1977; Kimmich, 1990). The scraping method is also used as a preparatory step in making intestinal homogenates and S9 fractions (Sinko and Hu, 1996; Sohlenius-Sternbeck and Orzechowski, 2004). The steps in preparing the small intestine for scraping are quite similar to the elution method. The rats can be fasted before excising the intestinal tissue and scraping the intestine to ensure that the

intestine is relatively clear of any foodstuff. Then, either by anaesthesia or decapitation, the rat is sacrificed and a section of the small intestine is removed. The lumen is then flushed with an isotonic buffer and split open lengthwise so that the mucosal side can be gently scraped with either a metal spatula or a glass slide (Sinko and Ho, 1996; Sohlenius-Sternbeck and Orzechowski, 2004). By applying different pressure, different cells can be obtained, but the meaning of “gentle” scraping pressure is quite subjective, such that there is no standardized cell yield each time the procedure is performed.

2.1.1.3 Enzyme-mediated Elution method

The enzyme-mediated elution method is one of the more widely used methods of obtaining isolated cells other than scraping because the cells remain intact. The method of Weiser (1973) uses a chelating agent (citrate) to dissociate the mucosal cells from rat intestines and is further treated with 1.5 mM EDTA in a phosphate-buffered 0.9% NaCl solution without using any proteases or other enzymes. After the small intestine is excised from the rat and washed, a different buffer containing the chelating agents are incubated in the lumen for a certain amount of time and repeated to elute the cells (Weiser, 1973). During the incubations, the small intestine is gently tapped to loosen the cells (Bonkovsky et al., 1985). The first few incubations elute the villus cells while the next few incubations elute the mid-villus cells followed by the crypt cells (Weiser, 1973). This method allows researchers to study the location of the metabolizing enzymes in relation to the crypt-villus axis and also allows only the villus cells

containing the CYP450 enzymes to be isolated. By excluding the mid-villus and crypt cells, the concentration of the CYP450 enzymes is greater than if all the enterocytes were included in the isolation. After the incubations, the cells are centrifuged at around 500-700 *g* for three to five minutes (Watford et al., 1979; Oates et al., 1997) to concentrate the cells and to transfer the pellet to another solution containing nutrients for the cells to use (Mohri and Uesawa, 2001).

Bonkovsky (1985) modified the method of Weiser to yield cells whose microsomes contained higher CYP450 enzymes than the original procedure. Mohri and Uesawa (2001) uses a slightly modified method of Weiser where they added dithiothreitol (DTT) to one of the incubating solutions and during incubation, the small intestine was tapped several times to loosen the cells. Watkins et al. (1987) uses a slightly modified method of Weiser where phenylmethylsulfonylfluoride (PMSF) is added as a protease inhibitor. Many other methods of elution have been described, all with varying concentrations of chelating agents, salts, incubating times and temperatures.

Experiments that use isolated cells are typically used for studying metabolism of drugs because it is a very simple model. There is no need to take into account monolayer permeability (like Caco-2 cells, Ussing chambers, intestinal slices), or blood flow (intestinal perfusions), or having to add co-factors (like microsomes). Basically, the most labour-intensive and time-consuming aspect of the method is to isolate the cells and maintain their viability. Typically, having cell viability that is greater than 80% is considered a minimal requirement for cell preparations, some investigators even require a cell viability of at least

90-95% before the cell preparations are deemed suitable for use (Fix, 1996). The viability of the cells is most commonly determined by the Trypan dye exclusion (Fix, 1996). Equal aliquots of the cell suspension and of 0.4% Trypan Blue solution is allowed to mix for five minutes before cells are viewed and counted in under a hemocytometer (Sigma-Aldrich Co., 2009,). This method is also used to determine the proper concentration of the cells that is needed for subsequent chemical incubations.

2.1.2 S9 fractions

Intestinal mucosal S9 fractions can be obtained from the three cell isolation procedures mentioned above, or by direct tissue homogenization where the fragmented cells are centrifuged at around 9000 *g* for 20 minutes. The supernatant fraction containing the cytosol and microsomes is used, hence the name S9 fraction (Sohlenius-Sternbeck and Orzechowski, 2004). An advantage of using S9 fractions in metabolism studies is that it is very efficient because the compounds do not have to travel through a cell membrane in order to interact with the CYP450 enzymes (van de Kerkhof et al., 2007a) and that it can be frozen and used another time (Sohlenius-Sternbeck and Orzechowski, 2004). However, physiological concentrations of co-factors such as NADPH-generating system (GS) are not present and have to be added, and drug transporters are also not present. This means that S9 fractions are mainly used to study metabolism, and not permeation or drug transport (van de Kerkhof et al., 2007a). Sohlenius-Sternbeck and Orzechowski (2004) studied the metabolism of

testosterone in intestinal S9 fractions and discovered that the metabolic activities and of glutathione transferase (GST) and phenol sulfotransferase (pST) are generally much lower than the liver S9. Liquiritigenin (a drug in preclinical studies for treating inflammatory liver disease) was also shown to be metabolized in rat intestinal S9 fractions (Kang et al., 2009).

2.1.3 Microsomes

Intestinal microsomes are obtained by further centrifuging S9 fractions at 12,000 *g* for 30 minutes, the supernatant decanted, filtered through two layers of cheesecloth and centrifuged at 100,000 *g* for 1 hour (Fasco et al., 1993). Poet et al. (2003) centrifuged their intestinal homogenates at 105,000 *g* for 90 minutes, then sonicated the supernatant for four-10 second intervals and centrifuged the solution at 105,000 *g* for 60 minutes according to the method used by van der Hoeven and Coon (1974). Walker et al. (2006) first centrifuged the homogenized cells at 10,000 *g* for 10 minutes to remove excess or undisrupted tissue and fat. Next, they centrifuged the supernatant at 15,000 *g* for 15 minutes to remove nuclei and mitochondria. Then the post-mitochondrial fraction was centrifuged at 100,000 *g* for 60 minutes at 4°C to pellet down the microsomes. The microsomes were frozen in liquid nitrogen until they were used.

The advantages of using microsomes in metabolism studies are that the drugs are directly exposed to the metabolizing enzymes, but co-factors also needed to be added. The limitations to using microsomes are similar to S9 fractions, such as physiological amounts of co-factors are not present, nor are

cytosolic DMEs and drug transporters (van de Kerkhof et al., 2007). In addition, the time and labour involved in preparing microsomal fractions is significant.

Another method of studying epithelial cell metabolism is to use immortalized or tumour intestinal cell lines. These tumour cells are derived from human colon tumour cell lines (Leibovitz et al., 1976) and are widely used for studying membrane transport (Werdenberg et al., 2003), sorption (Vasiluk et al., 2008) and recycling (Hochman et al., 2000). The advantages to using these tumour cell lines (e.g. Caco-2 cells) are that they do exhibit CYP 1A, 1B and 3A in the cells and they exhibit well-developed microvilli and form tight monolayers with a polarized distribution of brush border enzymes (Cavret and Feidt, 2005). However, the amounts of CYP enzymes may differ in Caco-2 cells versus rat intestinal cells. In Caco-2 cells, the levels of CYP3A4 are lower than in human intestinal tissue (Fearn and Hirst, 2006). In addition, Caco-2 cells are derived from human colon tumour cells whereas the focus is on *intestinal* cells.

3: METHODS

3.1 Overview

The small intestines of two male Sprague-Dawley rats (250-350 g each) were excised and the first 50 cm of the intestine was used to obtain enterocytes. After the cells had been isolated by using a chelating agent, the viability of the cells was verified by using Trypan Blue Dye Exclusion. The cells were then incubated in a 37°C water bath along with two test chemicals, BaP and DEP. At predetermined time intervals; the reaction was stopped by adding ice-cold hexane. Afterwards, an internal standard was added (PCB-153), and a portion of the hexane was placed in another vial to be analyzed by the GC-MS.

Cytochrome P450 1A enzymatic activity was also analyzed using Promega's P450-Glo™ Assays by measuring the luminescence generating from the transformation of luciferin-CEE by CYP1A in the enterocytes.

3.2 Chemicals and Animals

3.2.1 Chemicals

All of the chemical reagents and solvents used in the experiments are as follows with CAS no., chemical grade and vendor name in parenthesis,

SOLVENTS: acetonitrile (75-05-8, 99.8%, Caledon Laboratory Ltd., Georgetown, ON, Canada); hexane (110-54-3, >95%, Sigma-Aldrich, St Louis, MO, USA);

BUFFERS: potassium chloride (7447-40-7, 99.5%, EMD Chemicals Inc, Darmstadt, Germany.); sodium chloride (7647-14-5, unknown, The Canadian Salt Company Ltd., Pointe-Claire, PQ, Canada); sodium citrate (68-04-2, 99.5%, BioShop Canada Inc., Burlington, ON, Canada); monopotassium phosphate (7778-77-0, 99%, Caledon Laboratory Ltd.); dipotassium phosphate (7758-11-4, 98%, Anachemia Canada Inc, Montreal, QC, Canada); disodium hydrogen phosphate (7558-79-4, Caledon Laboratory Ltd.); phenylmethanesulfonyl fluoride (PMSF, 329-98-6, Sigma-Aldrich); ethylenediaminetetraacetic acid (EDTA, 60-00-4, >99.5%, BioShop Canada Inc.); dithiothreitol (DTT, 3483-12-3, >99%, Sigma-Aldrich); glucose (dextrose, 50-99-7, EMD Chemicals Inc, Gibbstown, NJ, USA), L-glutamine (56-85-9, 200 mM solution in 0.85% NaCl solution, BioWhittaker, Lonza, Walkersville, MD, USA); Minimum Essential Medium Eagle (MEM, cat. M4642, Sigma-Aldrich), sodium hydrogen carbonate (144-55-8, 99.7-100.3%, EMD Chemicals Inc., Darmstadt, Germany); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 7365-45-9, >99.5%, BioShop Canada Inc., Burlington, ON, Canada); collagenase (9001-12-1, cat. C5138, Sigma-Aldrich); **CHEMICALS:** Benzo[a]pyrene (BaP, 50-32-8, >98%, Sigma-Aldrich);

diethyl phthalate (DEP, 84-66-2, Sigma-Aldrich); PCB-153 (35065-27-1, 99.5±4%, Chromatographic Specialties Inc., Brockville, ON, Canada); sodium azide (26628-22-8, 99%, cat. B30111, BDH Inc, Toronto, ON, Canada); 0.4% Trypan blue solution (72-57-1, 0.4%, Sigma-Aldrich); **NADPH-GS**: glucose-6-phosphate, disodium salt hydrate (G6P, 3671-99-6, ≥ 98%, Sigma-Aldrich); β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADP, 2646-71-1, 96%, Sigma-Aldrich); glucose-6-phosphate dehydrogenase , type VII: Baker's Yeast, Crystalline suspension in 3.2 M (NH₄)₂SO₄ (G6PD, 9001-40-5, 13.2 mg_{protein}/mL, Sigma-Aldrich); and magnesium chloride (MgCl₂, 7786-30-3, ≥ 98%, Sigma-Aldrich).

3.2.2 Preparation of the Buffers and Dosing Chemicals

The buffers were prepared the day before the incubation and kept in a fridge overnight. The contents of the intestinal wash are as follows: 0.15 M NaCl, 1 mM DTT and 0.23 mM PMSF.

The contents of Buffer A (cell incubation) were: 96 mM NaCl, 27 mM Na-citrate, 1.5 mM KCl, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄ and 0.23 mM PMSF (pH 7.4). The contents of Buffer B (cell dissociation) were: 109 mM NaCl, 2.4 mM KCl, 1.5 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 1.5 mM EDTA, 10 mM glucose, 5 mM glutamine, 0.5 mM DTT and 0.23 mM PMSF (pH 7.4). The contents of the MEM solution (cell storage) were: 10.6 g_{powder}/L_{water}, 4.2 mM NaHCO₃, 15 mM HEPES (pH 7.0). The solutions were aerated for 30 minutes before the intestinal tissues

were excised. After the intestinal tissues were excised, the buffers were continually kept on ice.

The stock solutions of BaP and DEP were prepared in acetonitrile (83.83 μM each). The internal standard (I.S.) stock solution (PCB-153) was prepared in toluene (10.22 μM). Sodium azide was dissolved in dionized water (30 mM). The NaN_3 solution was only used for the control cells for trial 1. Trials 2 and 3 used cells that were kept overnight as control cells.

3.2.3 Animals

Two male Sprague-Dawley rats (350-450g, Charles-River, Wilmington, MA, USA) were used per experiment. Animals were not fasted overnight and intestinal tissues were obtained between 9:00-9:30 am each time. Rats were sacrificed by anesthesia using isoflurane. The abdominal cavity was cut open and the heart muscle was cut to complete the exsanguination. The small intestine, liver and stomach were immediately removed (the liver and stomach were to be used by another researcher) and placed in ice-cold intestinal wash buffer.

The proximal 50 cm of intestine (duodenum and part of the jejunum) was excised and divided into approximate two 25 cm segments (2 segments/intestine) and ice-cold intestinal wash was rinsed through it to dislodge any debris. Buffer A was injected into each segment, the ends were clamped closed and incubated in 37°C in buffer A for 15 minutes.

3.3 Cell Collection

The contents of the intestines were discarded and replaced with ice-cold buffer B, and the ends were clamped closed. The intestines were placed on a glass plate on top of a layer of ice and were massaged and tapped several times for 4 minutes to loosen the top layer of cells. The contents of the intestine were collected and the procedure was repeated for two more times for 2 minutes each time. Approximately 10 mL of MEM solution was placed in the beaker that was used to collect buffer B and its contents after it was filtered through a 250 and 200 μM pore size mesh (Sefar Filtration Inc., Depew, NY, USA).

3.4 Cell Separation

The MEM solution containing buffer B and the cells were centrifuged at 1660 *g* for five minutes (Thermo Fisher Scientific CL2 Centrifuge, Waltham, MA, USA). The supernatant was aspirated and discarded. The mass was re-suspended in ice-cold MEM + collagenase so that the final concentration of collagenase was 0.6 mg/mL. The solution was pipetted for 5 minutes to loosen the cell mass and then it was centrifuged again at 1660 *g* for 5 minutes. The supernatant was aspirated off, replaced with ice-cold MEM solution (without collagenase), pipetted for 5 minutes and centrifuged again at 1660 *g* for 5 minutes. The previous step was repeated once more, taking extra care not to add too much ice-cold MEM solution or else the cells concentration will be too

dilute. Finally, the MEM solution with cells was filtered through a 200 μM pore size mesh to get rid of any remaining mucous and the solution was kept on ice.

3.5 Quantifying Cell Viability Using Trypan Blue Dye Exclusion

A 1:1 ratio of 0.4% Trypan Blue Solution was added to the MEM solution containing cells to determine cell viability. Live (cells that excluded Trypan Blue) and dead (cells turned blue) cells were viewed under a microscope (Carl Zeiss, Axioskop2 Plus) and pictures were taken (Zeiss, AxioCam MRm) using Axio Vision (v 4.7.1.0) software. The cells were counted in a hemocytometer (VWR Levy Counting Chamber, Hausser Scientific, cat. 15170-208) according to Figure 4):

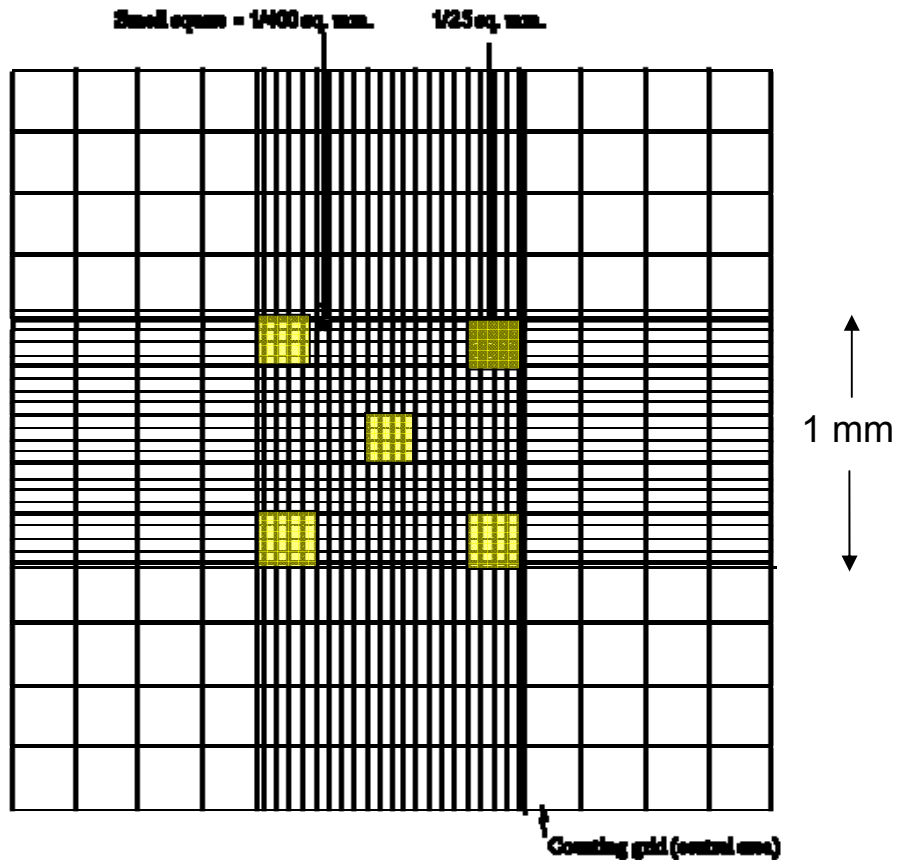


Figure 4. Example of grid lines on a hemocytometer (Caprette, 2007)

Briefly, the number of cells were counted in five $1/25 \text{ mm}^2$ square (four outer corners and the middle square) and averaged out. Since the depth of each square was 0.1 mm, the volume of each $1/25 \text{ mm}^2$ square was 0.004 mm^3 ($0.04 \text{ mm}^2 \times 0.1 \text{ mm}$). Since approximately $1000 \text{ mm}^3 = 1 \text{ cm}^3 = 1 \text{ mL}$, the cell concentration per mL was calculated as follows (equation 4):

$$\text{Average number of cells} \times \text{dilution factor} \times 250,000 = \# \text{ cells/mL}$$

4

In addition the number of stained cells was also counted to get the percent viability. For example, if the average number of total cells in all the squares was 30, then there would be approximately 15 million cells/mL. If there were 5 stained cells out of a total of 30 cells, then the percent viability would be 83% ($[(30-5)/30 \times 100]$).

3.6 *In vitro* Compound Delivery to Cells

After the cell concentration (per mL) was determined, the cell concentration was adjusted so that there were approximately 1 million cells per vial (volume 400 μ L; table 5). The time intervals for tests and controls were as follows: 0, 5, 10, 20, 60, 90, and 120 minutes. For trial 1, 5 μ L of the 0.3 mM NaN_3 solution was added to the cell solution so that the final NaN_3 concentration was 0.03 mM in the cell solution to inactivate cell metabolism. For trials 2 and 3, half of the volume of cell solution was split so that half would be kept overnight on the counter to reduce enzymatic activity to be used as control cells the next day (the experiment was run on two separate days). The cell solution was evenly split for the test and control runs so that 400 μ L of the cell solution was placed into a 2 mL screw-top amber auto sampler vial (Agilent Technologies Inc., Santa Clara, CA, USA) and capped with a screw cap with a Teflon/rubber/Teflon septum (Agilent Technologies Inc., Santa Clara, CA, USA). The dosing order of the cell solution and chemicals are listed in Table 5. Dosing order of solutions and chemicals

Table 5. Dosing order of solutions and chemicals

Order	Solution/Chemical to Add to Vial	Test Run (μL)	Control Run (μL)
#1	MEM solution with cells	400	400
#2	MEM solution only	100	95
#3	NaN ₃ solution*	-----	15
Incubate vials for 3 minutes in a 37°C water bath			
#3	Dosing chemical** in ACN	3	3
	Total volume***	503	503

* [NaN₃]_{Dionized water} = 30 mM

** [BaP]_{ACN} = 83.83 μM & [PCB-209]_{ACN} = 83.83 μM

*** Final concentration of BaP and PCB-209 in vial was 0.5 μM each; of NaN₃ was 0.03 mM.

After the vials were incubated in the water bath for 3 minutes, the reaction was initiated by adding 3 μL of the dosing chemical to the vial. The time was noted, the vials were capped immediately, shaken a few times by hand and placed on the roller system in the water bath (Grant orbital/linear shaking bat OLS200, Grant Instruments Cambridge Ltd., Cambridgeshire, UK) for a predetermined period of time. This step was repeated for the rest of the vials until all of them have been placed in the water bath.

The vials for test-0 minutes and control-0 minutes were performed last where after 3 μL of the dosing chemical was added, capped and shaken a few times by hand, and ice-cold hexane (1 mL) was added to the vial to stop the reaction. The vial was re-capped and vortexed for 5 seconds before being placed on ice.

At specific time points, 1 mL of ice-cold hexane was added to stop the reaction in other vials and placed on ice until all the vials were processed.

3.7 Chemical Extraction from Intestinal Cells

PCB-153 (40 μ L) was added to each of the vials to act as an internal standard (final concentration 0.39 μ M) and then all the vials were horizontally placed in a vortexer (VWR VX-2500 Multi-tube Vortexer, VWR International, LLC, West Chester, PA, USA) to be vortexed for 90 seconds (setting 6) to ensure that the hexane was completely mixed between the cells and was able to extract the compounds. The vials were then centrifuged at 2130 *g* for 5 minutes to separate the hexane/water phases and then 0.6 mL of the hexane supernatant was extracted and placed in a clean auto sampler vial for GC-MS analysis.

3.8 GC-MS Analysis

All the vials were analyzed using an Agilent 6890 gas chromatograph (GC) attached to an Agilent 5973N mass spectrometer (MS), with a programmable cool on-column injection port, a 30 m x 250 μ m x 0.25 μ m HP-5MS 5% phenyl methyl siloxane-coated column (Agilent), and a 5 m x 530 μ m x 0.25 μ m fused-silica deactivated guard column (Agilent). The oven temperature profile is shown in Table 6.

Table 6. Oven temperature profile for analyzing BaP, DEP and PCB-253.

Ramp	Temperature increase (°C / min)	Next temperature (°C)	Hold (min)	Runtime (min)
Start		60	4.00	4.00
Ramp 1	25	200	1.00	10.6
Ramp 2	20	300	5.00	20.6

The injection port and ion source temperatures were 60 and 230 °C, respectively. The carrier gas was helium at 1 mL/min flow rate. The ion selected for detection for BaP, DEP and PCB-153 were m/z 252, 149 and 498, respectively. The ions were selected based on properties of high intensity and low interference. The GC-MS injected 1 µL of the sample into the column by a 5-µL gas-tight glass syringe (Agilent). The height and area of the peaks corresponding to each of the chemical were integrated and quantified on the chromatogram using Chemstation (Hewlett Packard) software.

3.9 Quantifying Metabolites and Calculating Rates of Metabolism

3.9.1 Internal Standard

An internal standard (PCB-153) was added before the vortexing stage (30 mL of 57.85 M of PCB-153 dissolved in toluene) to ensure that it provided a consistent standard for the test chemicals because inconsistent volumes of hexane may be extracted from each vial. In addition, the sensitivity of the GC-

MS vary for each run and the sensitivity at the beginning of the run may differ substantially from the end of the run, especially if there are many vials to run. The ratio of the amount of analyte over the internal standard (analyte/I.S.) can provide a more accurate representation of the relative change in concentration of the test chemicals while taking into account the variation in volume of hexane extracted and GC-MS conditions.

3.9.2 Quantifying Test Compounds

An example of a chromatogram showing the peaks of BaP, PCB-209 and PCB-153 are shown in Figure 5. The peak height and area were quantified using Chemstation software. The height and area of the peak of each chemical is correlated with the quantity of chemical that was extracted with hexane.

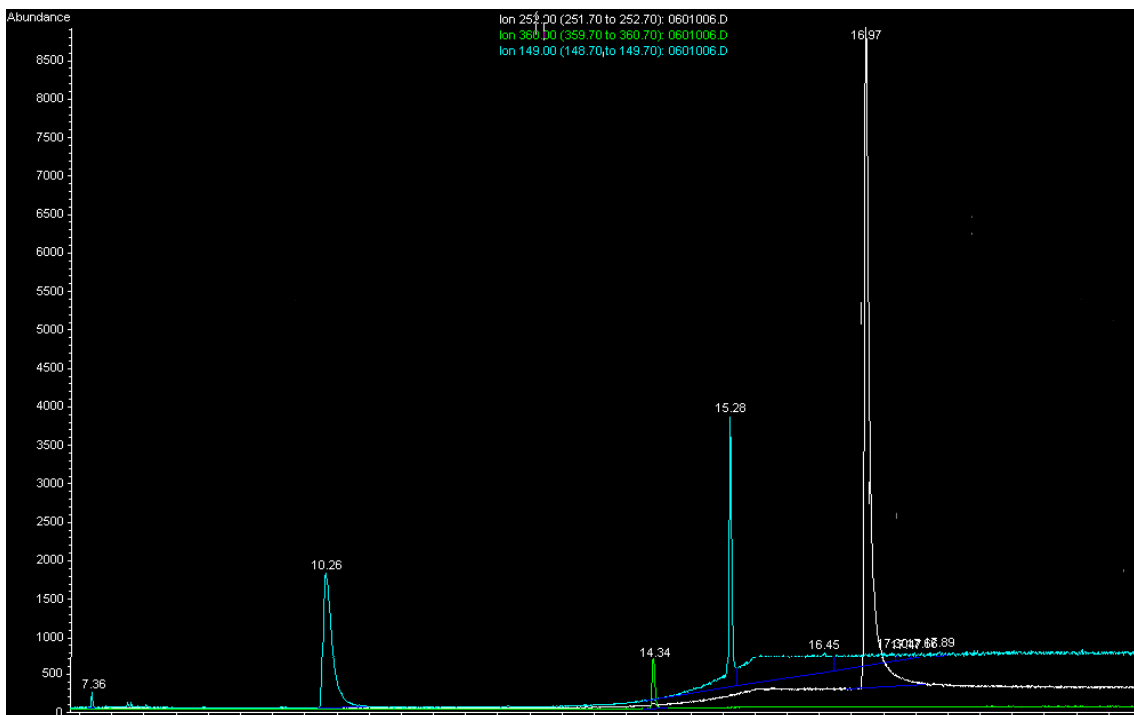


Figure 5. Example of chromatogram with BaP(16.97 min), DEP (10.26 min) and PCB-153 (14.34 min) peaks. The peak at 15.25 min is Di (2-ethylhexyl) phthalate, a widespread contaminant.

3.9.3 Calibration Curves

Calibration curves using BaP, DEP and PCB-153 (as the internal standard) were used to calculate the concentration of chemical.

Calibration concentrations of BaP and DEP were: 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μM in hexane. The concentration of PCB-153 remained at 0.1 μM in all seven vials.

A plot of the area ratio (analyte/I.S.) for trials 1 and 3 were plotted against the concentration of the analyte whereas a plot of the area abundance instead of the area ratio was used in trial 2. The linear regression of the data points was used to calculate the concentration of the dosing chemicals (BaP and DEP) that

were detected by the GC-MS. The formula of the linear regression is in the form “ $y = mx + b$ ”, where “ y ” was the area ratio (analyte/I.S.) and “ x ” is the value of the concentration of the analyte remaining in the experimental vials (the value we want to find, μM).

3.10 Cytochrome P4501A Enzyme Detection

3.10.1 Buffers

The presence and amount of CYP1A enzymes were detected in enterocytes and S9 liver fractions isolated from the same rat. S9 liver fractions were thawed from -70°C and were used as a positive control for the presence of CYP1A enzymes (when NADPH-GS was added) and were also used to compare relative amounts of the enzyme in enterocytes and in S9 liver fractions. S9 liver fractions without NADPH-GS added were deemed the negative controls because the microsomes require co-factors to become active (van de Kerkhoff, 2007a). The enterocytes were kept in an ice-cold MEM buffer whereas the S9 fraction was kept in a phosphate buffer (0.078 M KH_2PO_4 , 0.122 M K_2HPO_4 in deionized water) and 1.15% KCl solution on ice. The NADPH-GS was made the day of the experiment according to the following amounts: 0.08 M G6P, 7.44 mM NADP, 1.95 $\mu\text{L}/\text{mL}$ G6PD and 0.04 M MgCl_2 . The ratios of the S9 fraction (positive control) containing NADPH-GS, phosphate buffer, 1.15% KCl, and S9 was 1:2:1:1, respectively. The ratios of the S9 fraction (negative control) containing phosphate buffer, 1.15% KCl and S9 was 3:1:1, respectively. A total volume of 100 μL of the positive and negative control (each) of the S9 solution was prepared.

3.10.2 Cytochrome P4501A Enzyme Detection

The verification of the presence of active CYP4501A enzymes in enterocytes was conducted using Promega's P450-Glo™ Assays (Madison, WI, USA, Cat # V8751). More detailed instructions for using P450-Glo™ Assays can be found online at: www.promega.com/tbs/. Very briefly, the procedure for detecting CYP1A enzymes in enterocytes was as follows: After isolating the enterocytes, the cell solution was diluted so that there were approximately 2 million cells/ mL. According to Table 7, the total volume of the commercial luminogenic CYP substrate (luciferin-CEE) was determined. The cell concentration and the luciferin-CEE concentration was twice that of the final concentration (1 million cells/mL and 30 µM, respectively) because equal volumes of each were added in each vial. The final volume (per vial) was 100 µL.

For the S9 liver fraction vials, they were pre-mixed with amounts of the phosphate buffer, KCl buffer and NADPH-GS (if needed) according to the ratios mentioned in section 3.10.1. For each vial, 50 µL of the S9 liver solution (including its buffers and NADPH-GS) was added into a vial of which 10 µL comprise the S9 liver fraction.

Control vials contained cells that were prepared by leaving a small amount of the freshly isolated enterocytes at room temperature for one day so that all the enzymatic activity declined before the assay. "Buffer only" vials were used as a second "buffer" control, S9 fractions from hepatocytes (from the same rats) were used as a positive control (with NADPH-GS added) and S9 fractions without

NADPH-GS added were used as a negative control. Lastly, ACN (3 μ L per 500 μ L of cell solution) was added to account for the presence of the dosing solvent in the cell solution.

The cell solution and luciferin-CEE were incubated in a 37°C water bath for three hours in the dark. The luminescence of each vial was then recorded four times (known as “before” values) and 100 μ L of the detection reagent was added. Each vial was left at room temperature in the dark for another 20 minutes and then a luminescence reading of each vial (known as “after” values) was taken four times and the mean reading was reported. The ratio of the luminescence values (“after” values over “before” values) were calculated and normalized to luminescence per 1 million cells (enterocytes) or approximately 1 million cells (hepatocytes). The calculations for normalizing S9 liver fractions to number of cells is described in section 4.2.

Table 7. Volume and concentrations of various substrates used in detecting CYP1A enzymes in each vial.

Vial Category	#Cell/ mL	Volume of [cell] (μL)	[luciferin- CEE] (μM)	Volume of [luciferin- CEE] (μL)	Final [luciferin- CEE] (μM)	Final Volume (μL)
Live Cells	2×10^6	50	60	50	30	100
1-week old cells	2×10^6	50	60	50	30	100
Buffer Only (MEM)	None	50	60	50	30	100
Hepatocytes (S9) (+NADPH-GS, positive control)	N/A	50	60	50	30	100
Hepatocytes (S9) (no NADPH-GS, negative control)	N/A	50	60	50	30	100
Live Cells + ACN ($<0.6\%$v/v)	2×10^6	50	60	50	30	100

3.11 Calculating luminescence

According to Table 7, the vials were incubated in a 37°C water bath for three hours after equal volumes of the cell solution and luciferin-CEE was added to a vial. After incubating the solution for three hours, luminescence readings were taken with a luminometer (Turner Designs 20/20, CA, USA, delay: 2 sec, integration: 10 sec, standard mode) for four times per vial and averaged out.

These readings were designated as the “before” reading. Next, 100 µL of the luciferin detection reagent was added to the vial (bringing the total volume to 200 µL). The reaction was allowed to continue for 20 minutes at room temperature in a darkened room. After 20 minutes, four “after” luminescence readings were taken per vial and averaged out.

To compare the relative amounts of CYP1A enzymes as reflected by the amount of luminescence released from the reaction between luciferin-CEE and the luciferin detection reagent, the ratio of the averages of the “after” luminescence readings over the average “before” luminescence readings were calculated and plotted on a bar graph. The ratios of the “after” and “before” luminescence readings were used to take into account the luminescence variations among vials before the addition of the luciferin detection reagent.

3.12 Data Analysis

3.12.1 Depletion Rates

Assuming that analyte depletion rate follows first-order kinetics, the depletion rate can be represented as:

$$d[C]/ dt = -kC \qquad 5$$

Where C is the concentration of the analyte (moles/L) , t is time (minutes or hours) and k is the depletion rate constant (minute⁻¹).

If equation 5 is integrated on both sides and rearranged according to equation 6, then:

$$\int \frac{d[C]}{C} = \int -kt$$

$$\ln [C] - \ln [C]_0 = -kt$$

$$\ln [C] = -kt + \ln [C]_0 \quad \mathbf{6}$$

The natural logarithm was calculated for each concentration and plotted on a graph. The linear regression of the natural logarithm of the concentration versus time for control and test vials for each analyte was conducted using Microsoft's Excel©.

A Student's t-test (equation 7) was used to determine statistical differences between the depletion rates measured in the control and test samples. The Student t-test is a statistical hypothesis test that compares the means of two normally distributed populations. If the slopes of the control and test vials were not significantly different ($p > 0.05$), then the null hypothesis holds true and the biotransformation rate was either non-existent or too small to be detected by this experimental method.

$$\text{Student t-test} = \frac{|X_{test} - X_{ctrl}|}{\sqrt{(SE_{test})^2 + (SE_{ctrl})^2}} \quad \mathbf{7}$$

. The linear regression function of the natural logarithm of each concentration was performed on test and control lines. The summary output for the linear regression statistics gave the degrees of freedom for the test and control vials, and the standard error for the slope that is calculated in Student's T-test formula.

Where x_{test} and x_{ctrl} are the slopes of the linear regression lines of the natural logarithm of the concentrations of the analytes, and SE_{test} and SE_{ctrl} are the standard errors calculated from the linear regression function in Excel. The Student T-test value was compared to its value in a table according to its degree of freedom to test whether the test and control slopes were significantly different ($p < 0.05$). If the Student T-test value is greater than the one in the table, then the slopes of the test and control vials are significantly different ($p < 0.05$).

All of the time points were used in calculating the significant difference between the test and control slopes for BaP, but only time points up to 20 minutes were used for DEP. This was because there appeared to be a biphasic biotransformation rate for DEP and the initial period of the incubation period is expected to be the most representative of intestinal biotransformation rates.

4: RESULTS AND DISCUSSION

4.1 Cell Yield

Approximately 210 ± 112 (SD) million cells were isolated from the first 50 cm of the rat small intestine (duodenum and jejunum) per trial for the three trials. The average cell viability was 85.7 (± 1.9) per cent. The cell viability of the three experiments were: 83.5%, 86.6%, and 87%, respectively.

It was difficult to separate the cells in the solution completely because there was mucous in the solution. Prior to adding collagenase and filtering the cell solution three times (twice using the 250 μm pore mesh and once in 200 μm pore mesh), the cells appear clumped together when viewed under the light (Axiocam) microscope (Figure 6).

Under the microscope, there appeared to be darkened splotches, which were the mucosal masses that were stained with Trypan Blue solution. The circular objects were enterocytes. These dark splotches and the cells clumping together made it difficult to count the cells and calculate the cell viability. After altering the buffer solutions to the current method and adding 1.2 mg/mL of collagenase to the cell solution for 5 minutes, cell separation became much better (Figure 7). It was easier to count the number of live (clear) and dead (stained) cells in each square because they were nicely separated. Unfortunately, the Axiocam MRm photo-taking software does not capture pictures in colour, so it is difficult to see which cells were stained or not. The

slightly darkened mass on the right side of the figure would be a mass of mucous.

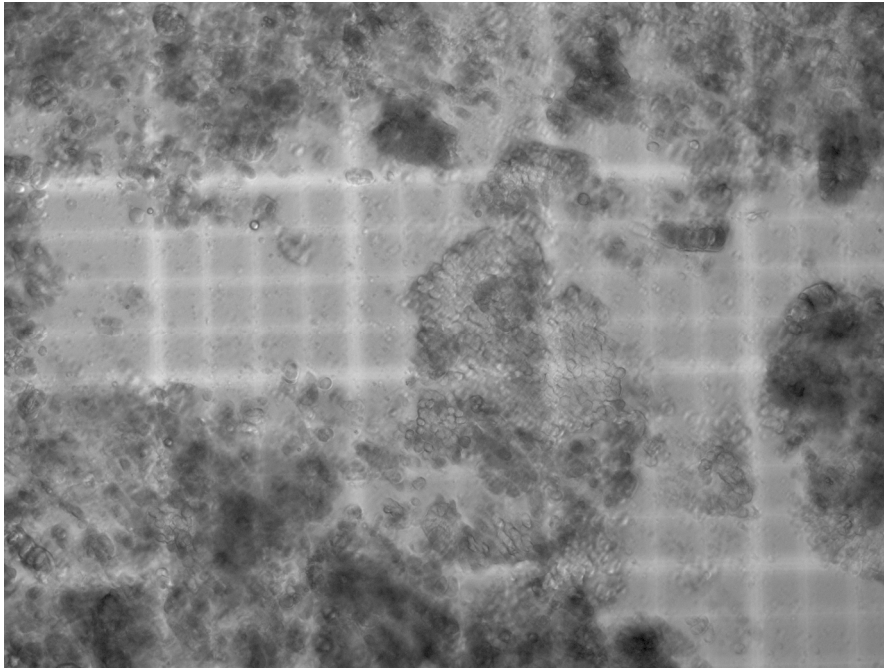


Figure 6. Isolated enterocytes when viewed under 10x magnification. Collagenase was not used to separate the cells. The thick white lines are 1 mm apart.

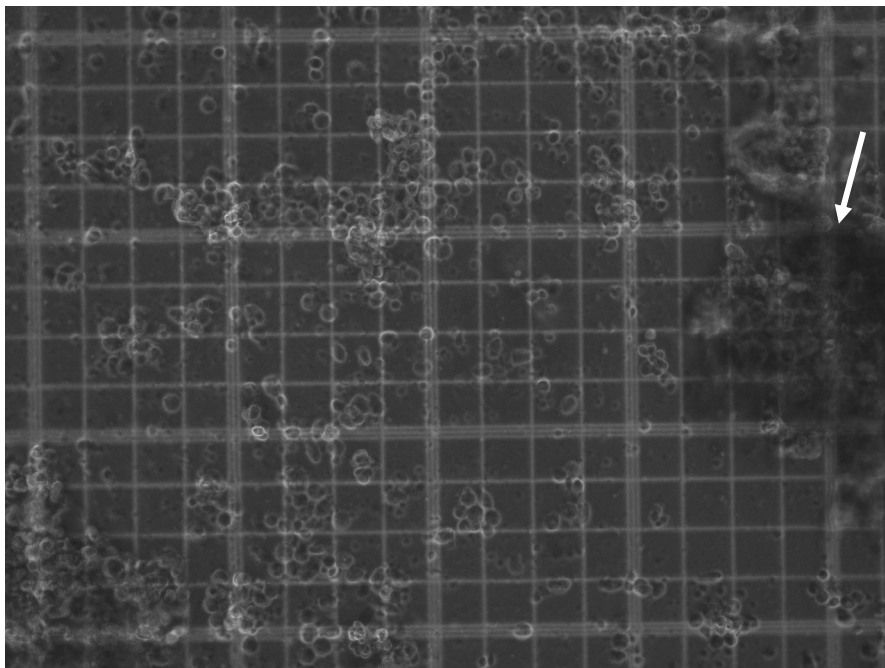


Figure 7. Isolated enterocytes when viewed under 10x magnification. Collagenase was used to separate the cells. The thick white lines are 1 mm apart. The white arrow shows a mucosal mass that is stained.

4.2 CYP1A1 Content

Using Promega's P450-Glo™ Assays, the amount of CYP1A1 enzymes was measured by incubating the cells with luciferin and measuring the luminescence before and after the addition of the luciferin detection reagent.

Figure 8 shows the luminescence values given for each category of vials (live cells, live cells plus ACN, control cells, buffer, S9 liver fractions with and without co-factors added) before the addition of the luciferin detection reagent. It can be seen that the luminescence values range between 0.01-0.04 (unitless).

Figure 9 shows the luminescence values after the luciferin detection reagent had been added for 20 minutes. It can be seen that the S9 liver fractions

with co-factors added has a luminescence that is approximately 28 times higher than live cells plus ACN, which has the next highest luminescence values. The luminescence values for control cells and buffer vials were between 0.12-0.88.

10 shows the relative luminescence ratios of the previously mentioned categories (live cells, live cells plus ACN, control cells, buffer, S9 liver fractions with and without co-factors added) in figure 8. The relative luminescence ratios are expressed to one million enterocytes or equivalent to one million hepatocytes.

Since S9 liver fractions are prepared from hepatocytes, it is quite simple to estimate the number of hepatocytes per mL of S9 liver fraction. The CYP1A1 activity in rat hepatocytes was not compared to rat enterocytes because the lab did not have a specific procedure for isolate rat hepatocytes.

The number of rat liver cells (hepatocytes) was calculated from a value given by Yung-Shan Lee (personal communication, a Ph.D student at the Gobas lab who is working on rat liver metabolism of several hydrophobic chemicals) when she was preparing rat liver S9 fractions. Yung-Shan Lee obtains her liver tissues the same time I obtain my intestinal tissues. Before she proceeds with her protocol for obtaining S9 liver fractions, she weighs each liver beforehand and measures the amount of S9 liver fractions she was able to obtain at the end. From 1 gram of liver, Lee (personal communication) obtained approximately 1.13 mL of S9 liver fraction. Lee measured approximately 14 g of liver in a 350 g rat. Houston (1994) stated that there are approximately 135 million hepatocytes per gram of liver. From the two previous values, I was able to calculate there were

approximately 1.2 million equivalent hepatocytes in the 10 μ L of S9 fractions with and without co-factors (10 μ L of S9 liver fraction per 50 μ L of S9 liver solution). In addition, I was able to calculate that there are approximately 1.89 billion hepatocytes per rat. Conversely, approximately 210 \pm 112 (SD) million cells were isolated from the first 50 cm of two intestines per trial for the three trials (data not shown). The amount of liver cells isolated from one rat is approximately nine times more than the amount of intestinal cells isolated from the first 50 cm of two intestines from two similar sized rats. This calculation is to illustrate that the metabolic capacity of the rat liver can be many times greater than that of the rat small intestine purely due to the number of metabolically active cells in each organ (assuming that all the cells in the liver and the first 50 cm of the small intestine contain metabolically active cells).

Figure 1010 shows that the CYP1A1 enzymatic activity of rat liver S9 fractions (even when normalized to an equivalent of 1 million cells), is 28 times higher than the enzymatic activity of live enterocytes incubated with 0.6% ACN. Although this test only measures the enzymatic activity of one enzyme, it shows that the CYP1A1 metabolic capability of the liver is many times greater than that of the small intestine.

Given that the S9 liver fractions without co-factors should have low enzymatic activity, it is encouraging to see that its enzymatic activity is lower than that of the live enterocytes and live enterocytes incubated in 0.6% ACN (luminescence ratio 104 versus 237 and 639, respectively). However, the enzymatic activity for the S9 liver fractions without co-factors is slightly higher

than control enterocytes and buffer (luminescence ratio 104 versus 7.35 and 19.5, respectively), which indicates that there is still some slight enzymatic activity in those S9 liver fractions.

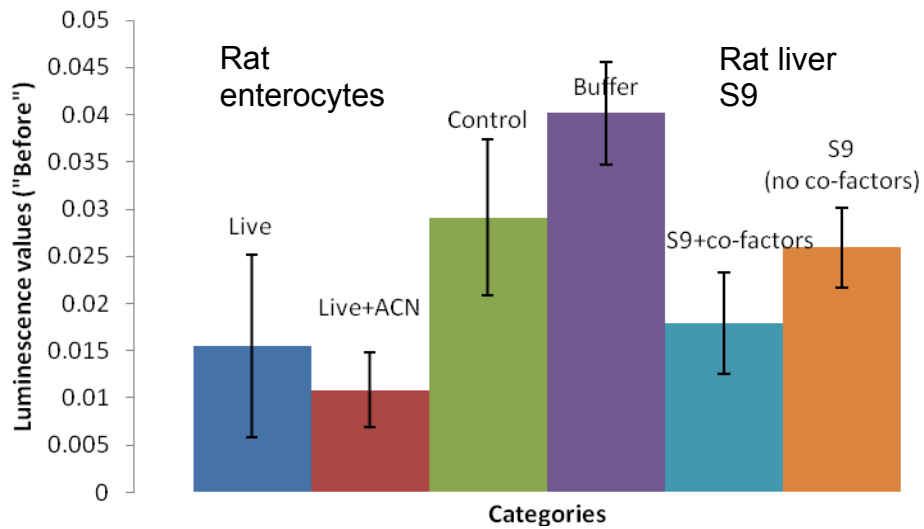


Figure 8. The "before" luminescence values (before the addition of the luciferin detection reagent) of live rat enterocytes (live), live rat enterocytes with ACN added (live + ACN), control rat enterocytes (control), buffer, rat liver S9 liver fraction with co-factors added (S9 + co-factors), and S9 liver fraction without co-factors added (S9, no co-factors). The vertical bars are the standard deviations.

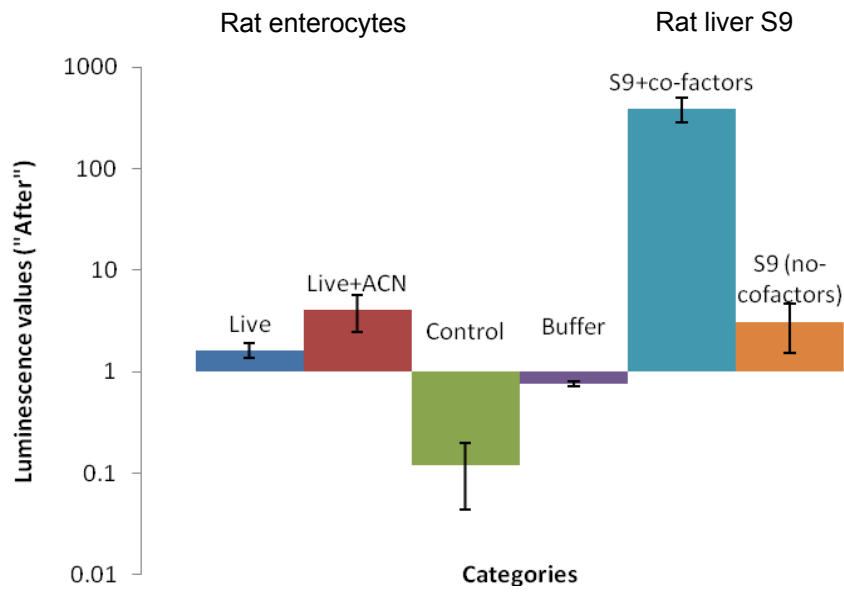


Figure 9. The "after" luminescence values (after the addition of the luciferin detection reagent) of live enterocytes (live), live enterocytes with ACN added (live + ACN), control enterocytes (control), buffer, rat liver S9 fraction with co-factors added (S9 + co-factors) and rat liver S9 fraction without co-factors added (S9, no-cofactors). The vertical bars are the standard deviations.

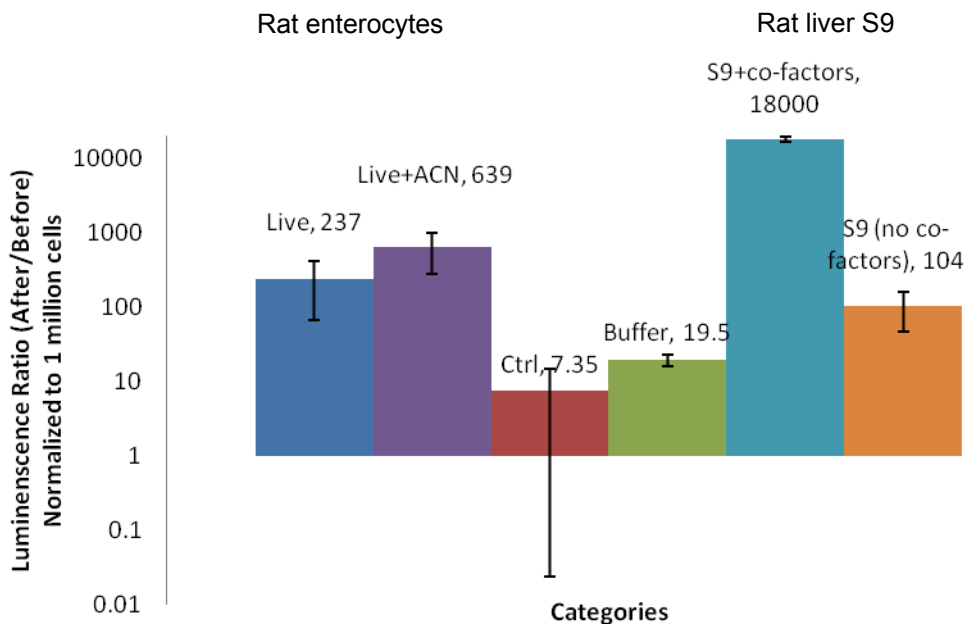


Figure 10. The relative luminescence ratios of the “after” to “before” luminescence values (normalized to 1 million enterocytes or hepatocytes) of live enterocytes (live), live enterocytes with ACN added (live + ACN), control enterocytes (control), buffer, rat liver S9 fraction with co-factors added (S9 + co-factors), and rat liver S9 fraction without co-factors added (S9, no-cofactors). The numbers above the bars are the luminescence ratio and the vertical bars are the standard deviation.

4.3 Calibration Curves

Figure 111 - 13 show the relationships between the concentration of each analyte (either BaP or DEP) and its corresponding area ratio (or area abundance in figure 11) for that concentration. The calibration curve of each analyte was calculated from the linear regression of each analyte (n = 3 for each analyte).

The conditions of the GCMS varied day to day so it was necessary to calculate calibration curves each time the experiment is run.

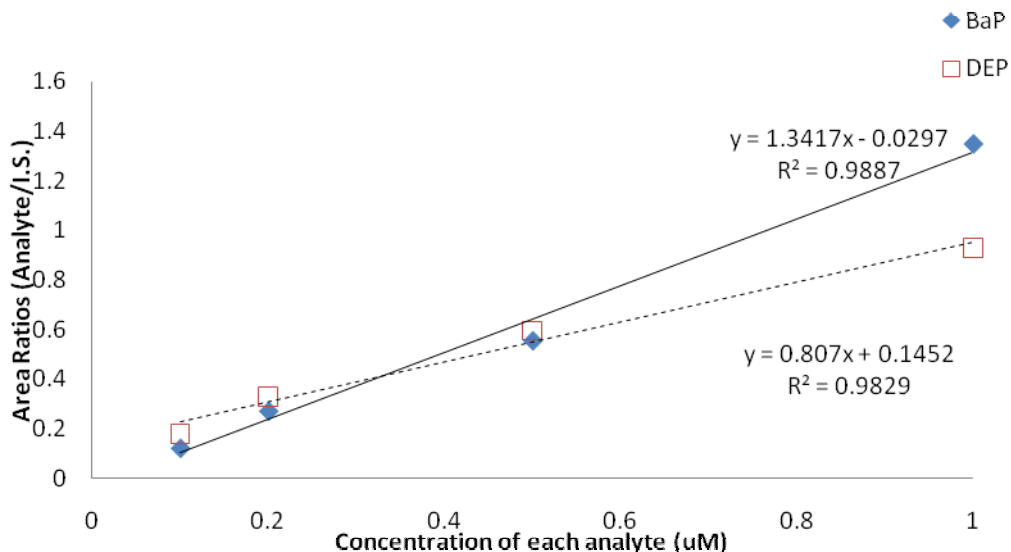


Figure 11. The relationship between the concentration of BaP and DEP versus its area ratio (area of analyte / area of I.S.) for trial 1. The linear regressions of each analyte are the calibration curves. The solid calibration curve represents BaP and the dashed calibration curve represents DEP.

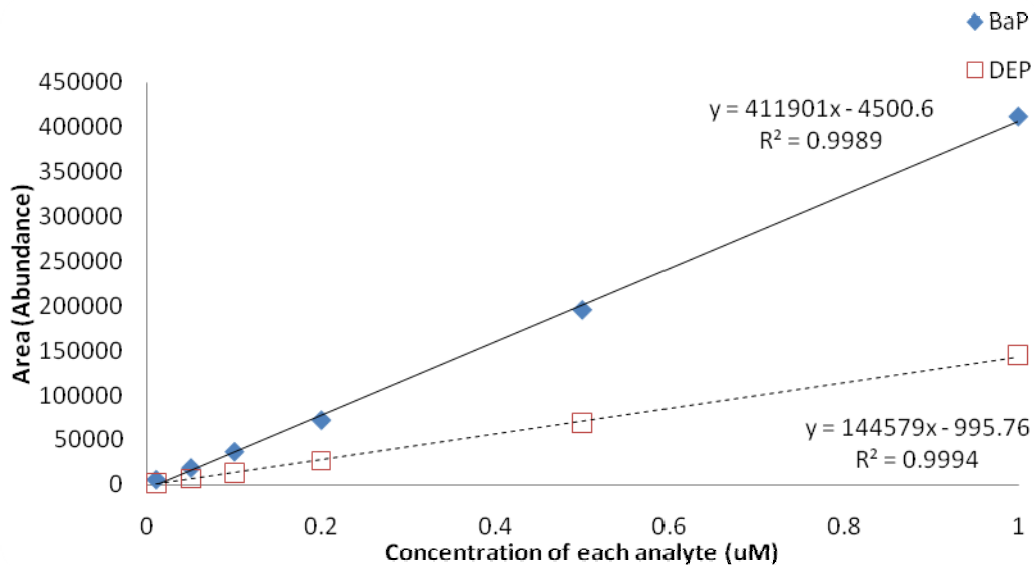


Figure 12. The relationship between the concentration of BaP and DEP versus its area ratio (area of analyte / area of I.S.) for trial 2. The linear regressions of each analyte are the calibration curves. The solid calibration curve represents BaP and the dashed calibration curve represents for DEP.

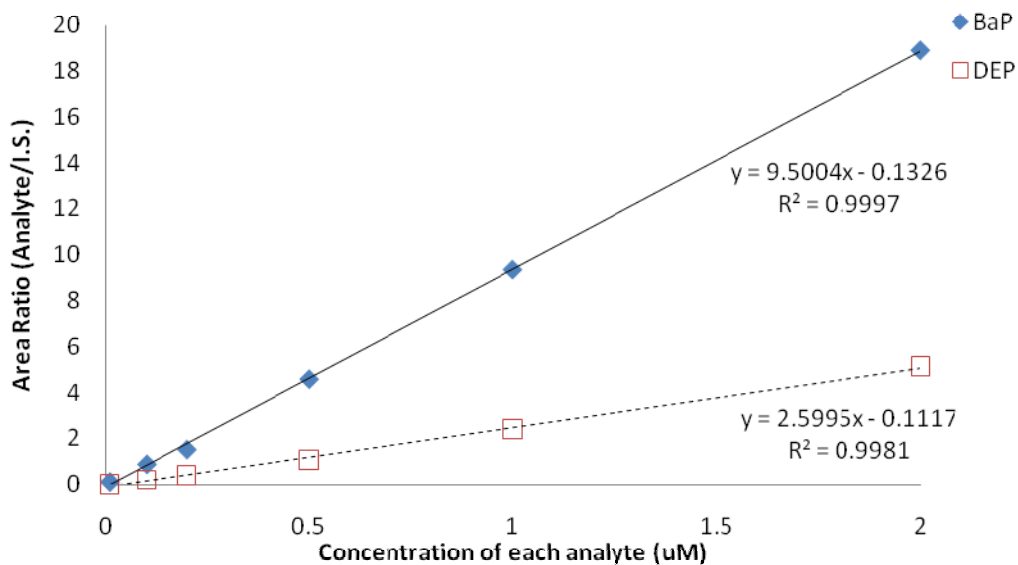


Figure 13. The relationship between the concentration of BaP and DEP versus its area ratio (area of analyte / area of I.S.) for trial 3. The linear regressions of each analyte are the calibration curves. The solid calibration curve represents BaP and the dashed calibration curve represents DEP.

4.4 Biotransformation Rates

4.4.1 Benzo[a]pyrene

Figures 14 - 16 show the natural log of the concentration of BaP remaining in the active (live) cell and inactive (control) cell vials over time. The concentration of BaP remaining in the vials in Figures 14 and 16 were calculated from the area ratio calibration curve of BaP (from Figures 11 and 13, respectively) whereas in Figure 15, the concentration of BaP was calculated from the area abundance peaks (Figure 12).

The linear regression lines of the natural logarithm of the concentration of BaP versus time in the control cell vials (dashed lines, Figures 11 - 13 'BaP

control slope') indicate a decrease in the amount of BaP that is extracted over the experimental period. A summary of the BaP control slopes for trials 1 -3 is listed in table 8.

Normally, one would expect that the concentration of BaP in the control vials would remain the same over time because it is presumed that the cells do not have any metabolic capabilities because they were left overnight on the counter in the lab. In an experiment performed by Vadi and colleagues (1975) in which she incubated BaP in rat liver cells to find what percentage of the unmetabolized BaP remained in the incubation medium, cytoplasm or the particulate cell fraction, she found that 23% of unmetabolized BaP was recovered in the incubation medium (60 g supernatant), 6% in the cytoplasm (105,000 g supernatant) and 70% was bound to the particulate cell fraction (105,000 g pellet). She centrifuged the incubation medium to separate out the cell components. This means that the majority of the unmetabolized BaP is most likely bound to the sedimentable cell fragments and may be difficult to extract using a solvent. In addition, Vadi was only able to recover approximately 93% of the BaP dose. This suggests that it is normal to extract less than 100% of the original dose.

As communicated by Danny Lee (MET student at the Gobas lab who was studying rat liver S9 metabolism of several PAHs, personal communication), when BaP was incubated in rat liver S9 fractions, a decrease in extraction efficiency was also seen. This means that observing a decrease in the amount of BaP extracted over time even in inactive cells (or rat liver S9 fractions) is quite

normal. Hawthorne and colleagues (2000) have noted that PAHs can become more tightly bound to an organic matrix over time and extracting PAHs from older samples may be more difficult. Therefore, it is normal to extract a smaller amount of BaP at the end of the experiment (after two hours) compared to at the beginning of the experiment (0 minutes).

The linear regression lines of the natural logarithm of the concentration of BaP versus time in live cell vials (solid line, figures 14-16, 'BaP test slopes') illustrate the observed depletion rates for BaP. Table 8 summarizes the slopes of the linear regression lines for test vials.

A Student's T-test showed that there was a significant difference between the BaP control and BaP test slopes for trial 1 ($p < 0.05$), but there was not a significant difference between the slopes for trials 2 and 3 ($p = 0.98$ and $p = 0.22$, respectively). Figures 15 and 16, corresponding to trials 2 and 3, show that there is large error associated with the test and control data points; such a large error reduces the statistical power of the Student T-test to observe for any significant difference between the BaP control and test slopes.

A difference between the BaP control and test slope was calculated to illustrate the *adjusted* depletion rate for trial 1 (table 8). This difference was taken because it was necessary to take into account the decrease in BaP concentration over time in the control cell vials. In trial 1, the adjusted depletion rate for BaP is $-4.3 (\pm 2.1) \times 10^{-3}/\text{min}$ (table 8).

Literature values on the biotransformation of BaP in the rat small intestine are rare. Researchers typically use the microsomal fraction of the rat small

intestine to measure for the presence of BaP metabolites (van de Wiel et al., 1992; Seitz et al., 1981; Chhabra et al., 1974; Salbe and Bjeldanes, 1984) or the fraction of certain BaP metabolites generated as a result of metabolism (Stoh et al., 1977). These values are quoted per gram of protein. As a result, it is difficult to compare values that are quoted as 'per gram of protein' versus 'per 10^6 cells' because no study has explicitly measured the amount of microsomal protein per intestinal cell. Experimenters also measured the rate of formation for certain BaP metabolites instead of the depletion of the parent compound for calculating the BaP metabolism. By counting only one or a few metabolites generated, only a partial picture of the total biotransformation rate emerges. For example, Stoh and colleagues (1977) studied BaP metabolism using rat intestinal cells and measured the amount of fluorescence produced by BaP monooxygenase. One BaP-monooxygenase unit is defined as the formation of 1 pmol of product/min using 3-hydroxy-BaP as the reference standard (Stoh et al., 1977). As can be seen from table 8, the adjusted biotransformation rate for BaP was normalized to 10^6 cells and the adjusted-normalized biotransformation rate for BaP was $-0.65 (\pm 0.31) \times 10^{-3}/\text{min}/10^6$ cells (trial 1). Stoh's value was converted into a biotransformation rate (appendix B) and the rate was approximately $-1.0 \times 10^{-5}/\text{min}/10^6$ cells. Stoh's BaP biotransformation rate is approximately 65 times less than the adjusted-normalized rate calculated in trial 1. A major difference between Stoh's experiment and this one was that he was only measuring the formation of one metabolite whereas this experiment measured the depletion of the parent compound. This difference in measurement may be a reason why

Stoh's rate is approximately 65 times less than the rate obtained from this experiment for trial 1. Miller and Ramos (2001) stated that many other metabolites are produced from BaP metabolism such as epoxides (2,3-, 4,5-, 7,8-, and 9,10-isomers), dihydrodiols (4,5-, 7,8-, 9,10-diols), phenols, and quinones (BaP 7,8-quinone). Stoh's method of measuring the formation of only one metabolite may not be the best way of measuring the biotransformation rate of that compound as the metabolites may be generated at different rates and amounts.

Another factor affecting the biotransformation rates of BaP would be its high lipophilicity and the problem of dissolving a high log K_{ow} molecule in an aqueous medium. Trying to introduce lipophilic compounds into an aqueous solution is very difficult because the compound has to be dissolved into an organic solvent first and then the solvent mixture has to be dissolved into the aqueous solution. The reason for not having a significant difference between BaP control and test slopes, and the large error associated with the slopes, may be because of its high log K_{ow} value. Benzo[a]pyrene is a very lipophilic compound, with a log K_{ow} of approximately 6 and as a result, it has a very high tendency to associate with any of the lipophilic membranes in the cell solution. Gerde and colleagues (1997) found that when dogs inhaled BaP into their trachea, the compound tended to be absorbed into the mucous and cell membranes first mainly because the lipophilic cells are dispersed in an aqueous (water and mucosal) medium. BaP has to travel through the mucous in order to reach the cell membranes. In this experiment, even after filtering the cells three times through a 250 μm and

200 µm pore size mesh, the mucous could not be completely separated from the cells. After introducing BaP into the cell solution, the compound could prefer to migrate into the cell membranes through the mucosal layer. If there is still some mucous surrounding the cells, then we would find BaP in the mucosal layer.

Miller and Ramos (2001) found that after BaP enters the cell, it may accumulate in the mitochondrion and nucleus instead of migrating into the microsomes where the CYP enzymes are contained. Much of the metabolic studies involving BaP use microsomes or S9 fractions where the problem of having mucous or cell membranes interfering with the compound interacting with the enzymes is less pronounced. This may be a reason why other studies were able to find a significant biotransformation rate for BaP whereas I was unable to find a significant difference between control and test runs for two of the three experiments.

Another factor that can affect the measurements of biotransformation rates are metabolites that are similar in structure to the parent compound are known to participate in end-product inhibition, where metabolites can compete with parent compounds for the same active site on enzymes, thus slowing down the biotransformation rate. Shen and colleagues (1979) found that metabolites of BaP (such as BaP 1,6-, 3,6- and 6,12-quinones) were found to be non-competitive inhibitors of BaP metabolism in the microsomes of rat livers. End-product inhibition may play a factor in affecting the biotransformation rates for BaP.

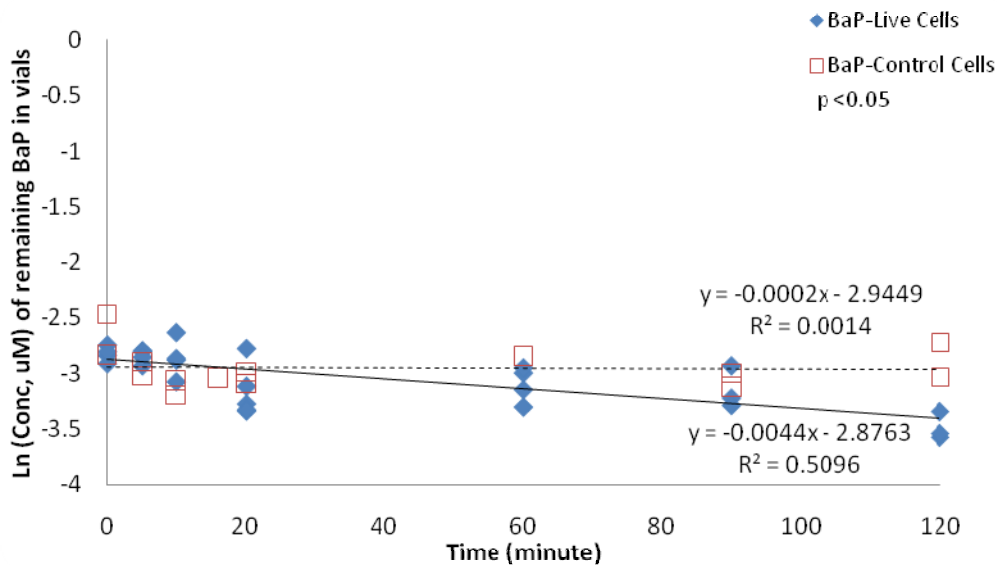


Figure 14. The natural logarithm of the concentration of BaP remaining in the live cells (◆) and control cells (□) vials over time for trial 1. The solid and dashed lines are the linear regression of the live and control cells, respectively.

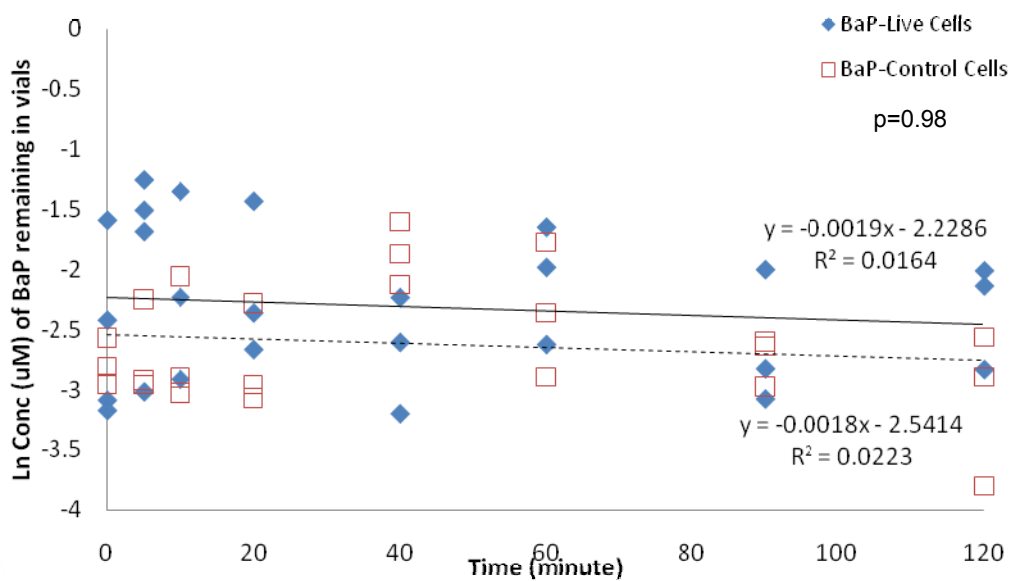


Figure 15. The natural logarithm of the concentration of BaP remaining in the live cells (◆) and control cells (□) over time for trial 2. The solid and dashed line are the linear regression of the live cells and control cells, respectively.

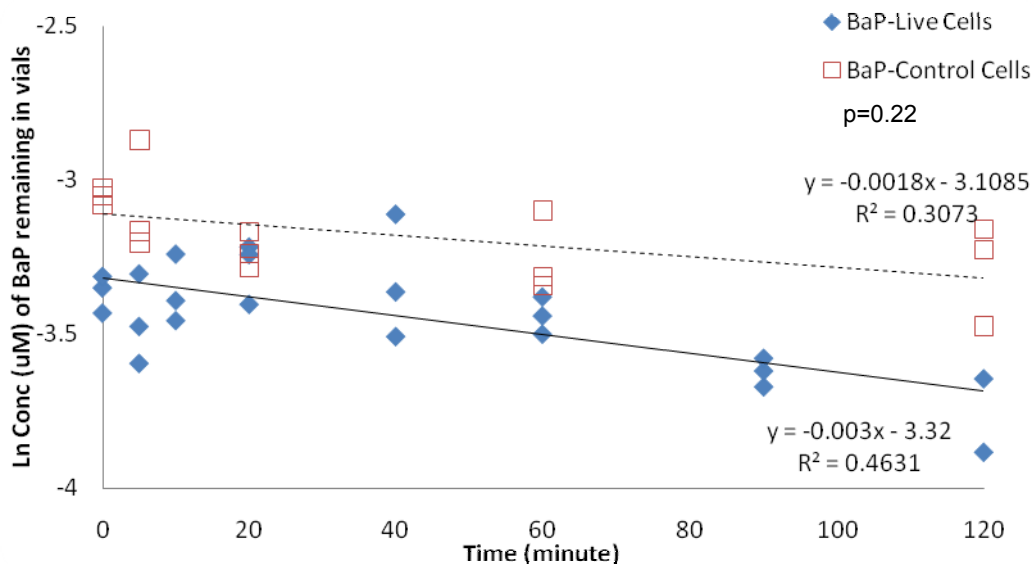


Figure 16. The natural logarithm of the concentration of BaP remaining in live cells (◆) and control cells (□) over time for trial 3. The solid and dashed lines are the linear regression of the live cells and control cells, respectively.

Table 8. Summary of adjusted and normalized depletion rates for BaP for trials 1-3. All of the errors are standard errors (S.E.)

BaP	Test Slope ($\times 10^{-3}/\text{min}$)	Control Slope ($\times 10^{-3}/\text{min}$)	Difference ($\times 10^{-3}/\text{min}$)	Cells/vial ($\times 10^6$ cells/vial)	Normalized ($\times 10^{-3}/\text{min}/10^6$ cells)	p-value (two-tailed)
Trial 1*	-4.4 (± 0.87)	-0.15 (± 1.2)	-4.3 (± 2.1)	6.6	-0.65 (± 0.31)	6.2×10^{-4}
Trial 2	-1.9 (± 3.0)	-1.8 (± 2.5)	-0.11 (± 5.5)	4.9	-0.02 (± 1.0)	0.98
Trial 3	-3.0 (± 0.71)	-1.8 (± 0.73)	-1.2 (1.4)	5.4	-0.24 (± 0.28)	0.22
			Apparent biotransformation/depletion rate = $-0.65 (\pm 0.31) \times 10^{-3}/\text{min}/10^6$ cells			

* The difference in slopes between the test and control cell vials was significantly different ($p < 0.05$).

4.4.2 Diethyl Phthalate

Figures 17 - 19 show the natural logarithm of the concentration of DEP remaining in the active (live) cell and inactive (control) cells. The linear regression of the natural logarithm of the concentration of DEP remaining in the control cells (dashed line, Figures 17 – 19, ‘DEP control slope’) show a decrease in the amount of DEP extracted over the experimental period. A summary of the DEP control slopes in Figures 17 – 19 are located in table 9.

Similar to BaP, the DEP control slopes in Figures 16 - 18 show a decrease in the amount of DEP extracted over time. It is not entirely certain why the amount of DEP extracted over time would decrease, although the reason may be similar to that of BaP; DEP becomes increasingly bound to cell fragments and becomes more difficult for hexane to extract it over time.

The solid line in Figures 17 – 19 shows the linear regression of the natural logarithm of the concentration of DEP remaining in test cell vials over time (‘DEP test slope’). The solid lines in Figures 17 – 19 shows a greater decrease in DEP concentration over time compared to the dashed lines (control cell vials). A Student’s T-test showed that there was significant difference between the DEP test and DEP control slopes ($p < 0.05$). Therefore, the *adjusted* depletion rates for DEP were calculated by taking the difference between the slopes of the DEP test and DEP control lines. These differences are summarized in table 9. It was necessary to calculate the difference between the DEP test and DEP control slopes because it took into account the decreasing amount of DEP that could be extracted in the *control* vials over time. If it was assumed that 100 % of the original DEP dose in control vials was extracted from 0 – 120 minutes, then the

DEP test slope would be artificially greater. In addition, the number of cells in trials 1 – 3 differs, so the adjusted depletion rates for DEP were normalized to 10^6 cells so that the rates for the three trials can be compared more easily. The normalized-adjusted depletion rates for DEP are listed in table 9 and the average of the normalized-adjusted depletion rates for DEP is $-0.049 (\pm 1.1 \text{ S.E.})/\text{min}/10^6$ cells.

One of the most widely cited papers that explicitly studied the hydrolysis of a few shorter-chain phthalates (dimethyl, diethyl, di-*n*-butyl phthalates) and longer-chain phthalates (dicyclohexyl, di-*n*-octyl, and di-(2-ethylhexyl) phthalates) in the rat small intestinal microsomes was performed by Rowland and colleagues in 1977. Rowland and colleagues (1977) found that the shorter-chain phthalates were hydrolyzed faster than longer-chain phthalates and it may have been due to their solubility in water. The biotransformation rate of DEP in the rat small intestine (figure 1 in the paper, calculations shown in appendix C) was estimated to be $-0.027 \text{ umol}/\text{min}/\text{g}_{\text{tissue}}$. Unfortunately, the rate was not measured as a function of the number of intestinal cells so a direct comparison cannot be made. The weight of the cells or the number of cells isolated per gram of intestinal tissue was not measured in this experiment because of the method used to elute the cells. It was not possible to separate the buffers and the intestinal cells because the cells needed to be immersed in the buffer to remain active and viable. What is important to remember is that smaller PEs are metabolized quicker than larger PEs.

Api's review (2001) focused extensively on the toxicological profile of DEP. In his review (from Ioku et al., 1976), he stated that after oral administration of DEP to rats, the majority of the dose accumulated in the kidneys and liver, and was detected within 20 minutes after oral administration. The detected DEP decreased to trace amounts within 24 hours. Ioku and colleagues (1976) found that the majority of metabolites were excreted in the urine (47% within 12 hours, 82% within 24 hours, and 90% within 48 hours), meaning that DEP has a short half-life in rats. Williams and Blanchfield (1975) also found that orally administered dibutyl phthalate (DBP; which has a similar structure to DEP) was rapidly metabolized in rats in which 80-90% of the dose was excreted in the urine after 48 hours.

A review conducted by Frederiksen and colleagues (2007) showed that phthalate diesters are metabolized in two steps; a phase I hydrolysis followed by a phase II conjugation. Phthalate diesters are first hydrolyzed into a monoester phthalate by lipases and esterases that can be found in the intestine and parenchyma. Lower MW phthalate diesters are excreted in the urine primarily as their monoester derivative. Higher MW phthalate diesters usually undergo a phase II conjugation in which the conjugation is catalyzed by the enzyme uridine 5'-diphosphoglucuronyl transferase and excreted in the urine (Frederiksen et al., 2007).

These three studies (Rowland et al., 1977; Ioku et al., 1976; and Williams and Blanchfield, 1975) and Frederiksen's review (2007) suggest that DEP metabolism in the rat is quite rapid and that the small intestine is capable of

metabolizing DEP. A significant deplete rate was found in this experiment using DEP after only two hours of incubation time. The half-life calculated from this experiment ($\ln(2)/(0.049/\text{min}/10^6 \text{ cells})$) is approximately 2.4 hours/ 10^6 cells.

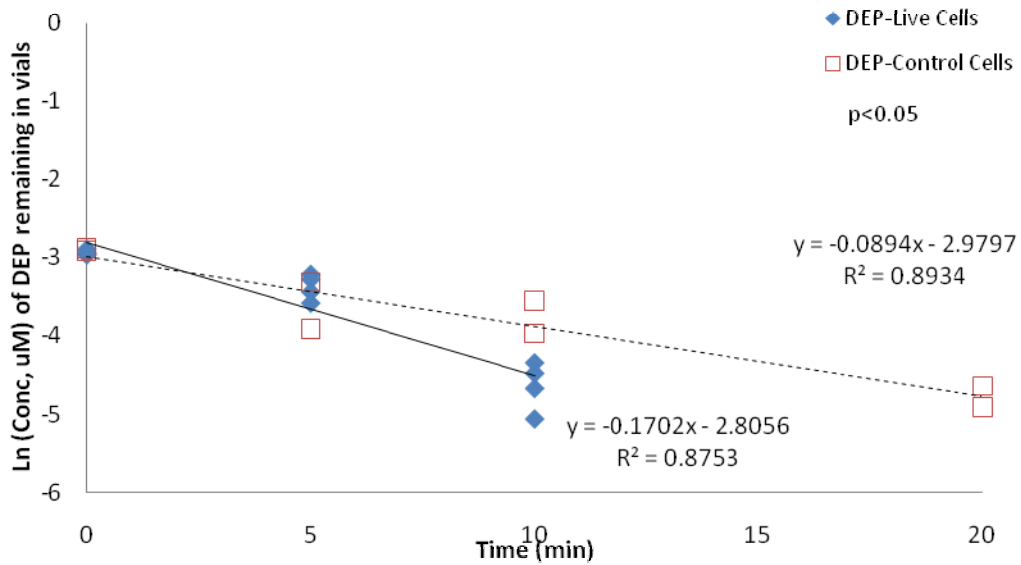


Figure 17. The natural logarithm of the concentration of DEP remaining in live cells (◆) and control cells (□) over time for trial 1. The solid and dashed lines are the linear regression of the live cells and control cells, respectively.

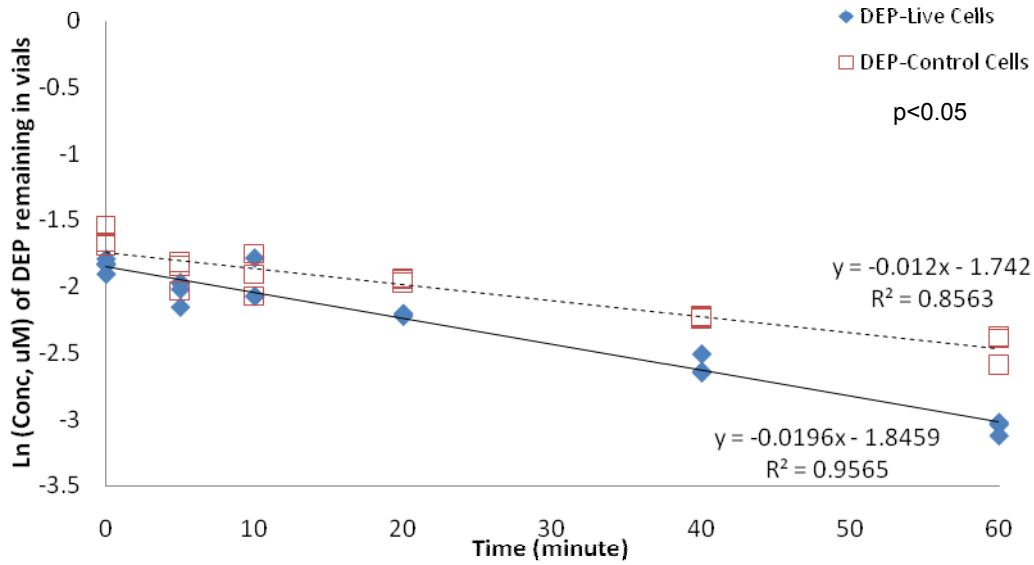


Figure 18. The natural logarithm of the concentration of DEP remaining in live cells (◆) and in control cells (□) over time for trial 2. The solid and dashed lines are the linear regression of the live cells and control cells, respectively

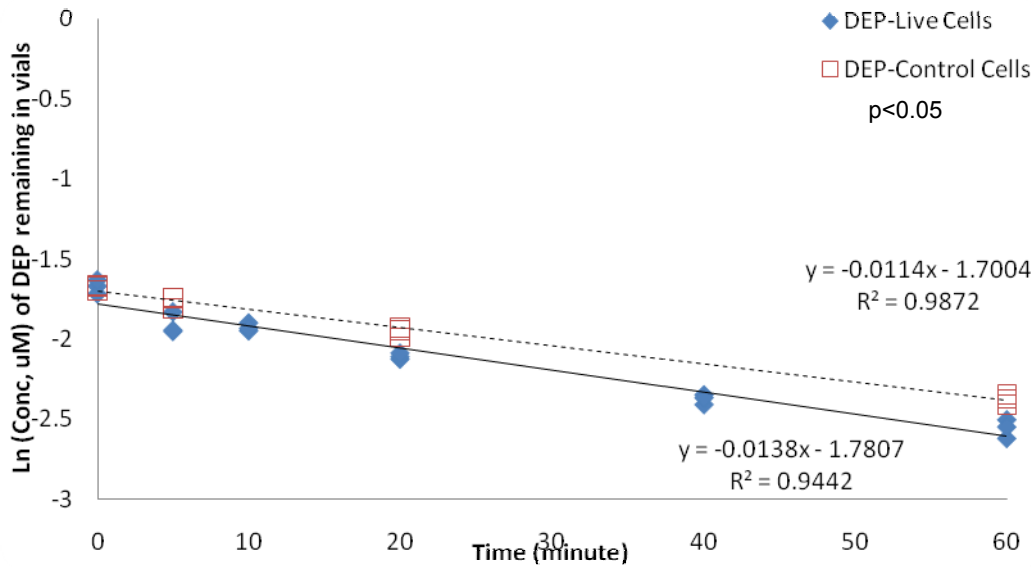


Figure 19. The natural logarithm of the concentration of DEP remaining in the live cells (◆) and in control cells (□) over time for trial 3. The solid and dashed lines are the linear regression of the live cells and control cells data points, respectively.

Table 9. Summary of adjusted and normalized depletion rates for DEP for trials 1-3. All of the errors are standard errors (S.E.)

DEP	Test Slope ($\times 10^{-2}/\text{min}$)	Control Slope ($\times 10^{-2}/\text{min}$)	Difference ($\times 10^{-2}/\text{min}$)	Cells/vial ($\times 10^6$ cells/vial)	Normalized ($\times 10^{-2}/\text{min}/10^6$ cells)	p-value (two-tailed)
Trial 1*	-17 (± 2.0)	-8.9 (± 1.3)	-8.1 (± 3.3)	6.6	-1.2 (± 0.49)	3.9×10^{-3}
Trial 2*	-2.0 (± 0.098)	-1.2 (± 0.12)	-0.80 (± 0.22)	4.9	-0.16 (± 0.044)	2.5×10^{-6}
Trial 3*	-1.4 (± 0.083)	-1.1 (± 0.041)	-0.30 (± 0.12)	5.4	-0.06 (± 0.024)	$<10^{-6}$
			Average apparent biotransformation/depletion rate: $-0.49 (\pm 1.1) \times 10^{-2}/\text{min}/10^6$ cells			

* There is a significant difference between the DEP test and control slopes ($p < 0.05$).

5: CONCLUSION

The average cell yield of intestinal cells isolated from the proximal 50 cm of the rat small intestine was $14 \pm 2.2 \times 10^6$ intestinal cells/ mL (n=3). The total cell yield was approximately $210 \pm 112 \times 10^6$ intestinal cells per two intestines (proximal 50 cm), which is substantially less than the number of liver cells that can be isolated from a single rat liver (approximately 1.89×10^9 hepatocytes, Yung-Shan Lee). The CYP1A1 activity of the liver S9 fraction is approximately 38 times greater than in the live intestinal cells plus ACN even after normalizing for the number of cells. The relatively smaller number of cells isolated from the small intestine compared to the liver, and the relatively smaller activity of CYP1A1 enzymes in the small intestinal cells compared to the liver cells makes it more challenging to conduct *in vitro* biotransformation rate studies. It makes it harder to detect slow biotransformation rates as was the case with BaP.

It was a labour-intensive process to isolate the cells, separate the mucus from the cells, and check for cell viability (Trypan blue dye exclusion). After excising the small intestines from the rat, the entire isolation procedure including checking for cell viability took approximately 2.5 to 3 hours. Given that it was not possible to completely separate the cells from the mucous, it may be easier to obtain an intestinal cell S9 fraction because less labour is involved and the sample contains more of the metabolizing enzymes rather than cell membranes and mucous.

The BaP test and control slopes for trial 1 (figure 13) was significantly different ($p < 0.05$) whereas there was no significant difference in trials 2 and 3 (figures 15 and 16). The difference between the BaP test and control slopes for trial 1 is $-4.3 (\pm 2.1) \times 10^{-3}/\text{min}$ and the normalized-adjusted depletion rate for BaP is $-0.65 (\pm 0.31) \times 10^{-3}/\text{min}/10^6$ cells (table 8). Due to the poor reproducibility of measuring BaP depletion rates in rat small intestinal cells, it was not possible to determine the variability in BaP depletion rate measurements so an average of the three trials cannot be calculated. The reasons for the poor reproducibility are unknown, but it may be because of BaP preferentially be bound to cell tissue rather than be extracted, or dissolving high lipophilicity compounds into an aqueous medium, or end-product inhibition.

There is a significant difference between DEP test and control slopes ($p < 0.05$, figures 7– 19) and after adjusting for the number of cells in each trial, the average normalized-adjusted depletion rate for DEP is $-0.49 (\pm 1.1) \times 10^{-2}/\text{min}/10^6$ cells (table 9).

It is difficult to compare the depletion rates of BaP and DEP in rat enterocytes with values found in literature because there are few studies that specifically look at the depletion rates. Often, researchers focus on the presence of metabolites and infer that metabolism is occurring. In addition, the majority of the researchers obtain microsomal or S9 fractions instead of isolating intact cells. This difference in cell/cell fraction preparation makes it difficult to compare depletion rates because values obtained from cell fractions are normalized to protein content whereas I normalized my values to the number of cells.

Isolating intestinal cells is probably not the best method for measuring biotransformation rates because it is a labour-intensive process; it requires at least 2.5 to 3 hours just to isolate the cells. Afterwards, it requires another 30 minutes to one hour to check and calculate cell viability, and then the cells can be used for the biotransformation studies. It was difficult to separate the mucous from the cells even after filtering the cell solution two times through a 250 μm pore mesh. Lastly, because the compounds may have to travel through the mucous and the cell membrane in order to make contact with the metabolizing enzymes, it introduces another obstacle to measuring slow depletion rates (such as BaP) and when test compounds are large in size such as active transport is needed for the compound to move across the cell membrane. However, using enterocytes for metabolizing studies is a more complete system because the cells are all intact and contain all of their metabolizing enzymes. Enterocytes also generate their own NADPH so no-cofactors are needed in order to activate the enzymes. The most important thing to consider when isolating enterocytes is to remove as much of the mucous as possible because it makes it easier to count the cells and removes a possible complicating factor in enterocyte metabolism.

APPENDICES

Appendix A

Table 10. List of POPs on the Stockholm Convention that includes pesticides, industrial chemicals and by-product contaminants (dioxins and furans) (Stockholm Convention on Persistent Organic Pollutants, 2009)

Initial 12 POPs	New 9 POPs
Aldrin	Chlordecone
Chlordane	Hexabromobiphenyl
Dieldrin	Lindane
Endrin	Alpha hexachlorocyclohexane
Heptachlor	Beta hexachlorocyclohexane
Mirex	Tetrabromodiphenyl ether and Pentabromodiphenyl ether
Toxaphene	Hexabromodiphenyl ether and heptabromodiphenyl ether
DDT	Perfluorooctane sulfonic acid (PFOS), its salts and perfluorooctane sulfonyl fluoride
Hexachlorobenzene (HCB)	Pentachlorobenzene
PCBs	
Dioxins	
Furans	

Appendix B

The calculated estimated biotransformation rate of BaP in rat small intestinal tissue from Stoh, S.J., Grafstrom, R.C., Burke, M.D., Orrenius, S. 1977. Benzo[a]pyrene metabolism by isolated rat intestinal epithelial cells. Arch. Biochem. Biophys. 179, 71-80.

Data estimated from figure 3, "Metabolism of BaP with time in isolated intestinal cells from control and 3-methylcholanthrene-treated rats". The initial BaP concentration in the incubation medium was 150 μM .

Time (min)	Estimated BaP-MO* (pmol product/ 10^6 cells)
0	0
2	6.7
4	12
5	15
6	17
7.5	22
8.3	23
10	28
15	37
20	43

*Benzo[a]pyrene-monoxygenase

The estimated amount of BaP-MO produced was subtracted from the initial concentration (150 μM), then the amount of unmetabolized BaP (that was remaining) was converted to its natural log values. The linear regression was calculated from the natural log values and the equation generated is: $y = -10^{-5} x + 5.0106$, $R^2 = 0.9706$, where "y" is the amount of BaP remaining (μM) and "x" is the time (minutes). The slope from the equation gives a depletion rate (k) of approximately $-10^5 / \text{min} / 10^6$ cells.

Appendix C

The calculated estimated biotransformation rate of BaP in rat small intestinal tissue from Rowland, I.R., Cottrell, R.C., Phillips, J.C. 1977. Hydrolysis of phthalate esters by the gastrointestinal contents of the rat. Food Cos Toxicol. 15, 17-21.

Data was estimated from figure 1, "Rate of metabolism of Dimethyl phthalate, diethyl phthalate and dibutyl phthalate when incubated at a final concentration of 1 mg/mL* with the contents of the rat small intestine (0.2 g/mL)".

*1 mg_{DEP}/mL = 4499.6 μM_{DEP}

Time (min)	% of DEP metabolized
0	0
2	2.0
5	4.0
10	5.5
15	9.0
20	11
30	15

The amount of DEP remaining was calculated by subtracting the percent of DEP metabolized and the natural log was calculated from that value. The linear regression of the natural log values was calculated, yielding the equation $y = -0.0053x + 8.4038$, $R^2 = 0.9899$ where "y" is the amount of DEP remaining (μM) and "x" is the time (minutes). The depletion (k) rate is estimated to be $-0.0265 \text{ umol/min/g}_{\text{tissue}}$.

Appendix D

An example on how the Student's T-test was used to test whether there is a significant difference between the slopes of the linear regression lines between the control and test cell vial data points for BaP in trial 1 (Table 11).

Table 11. Data values of the natural log of BaP concentrations versus time in trial 1.

m = 1.34 b = - 0.03							
Test vials for BaP				Control vials for BaP			
Time (Min.)	Area Ratios	Conc. (uM)	Ln Conc	Time (Min.)	Area Ratios	Conc. (uM)	Ln Conc.
0	0.06	0.06	-2.74	0	0.05	0.06	-2.83
0	0.04	0.05	-2.91	0	0.08	0.08	-2.47
0	0.05	0.06	-2.84	5	0.04	0.06	-2.89
0	0.05	0.06	-2.80	5	0.04	0.05	-3.02
5	0.05	0.06	-2.79	10	0.03	0.04	-3.20
5	0.05	0.06	-2.80	10	0.03	0.05	-3.06
5	0.04	0.05	-2.93	20	0.04	0.05	-2.99
5	0.05	0.06	-2.85	20	0.03	0.05	-3.09
10	0.05	0.06	-2.88	60	0.05	0.06	-2.84
10	0.07	0.07	-2.63	16	0.03	0.05	-3.04
10	0.05	0.06	-2.86	90	0.04	0.05	-3.00
10	0.03	0.05	-3.07	90	0.03	0.04	-3.12
20	0.05	0.06	-2.77	120	0.03	0.05	-3.03
20	0.02	0.04	-3.34				
20	0.02	0.04	-3.28				
20	0.03	0.04	-3.12				
60	0.04	0.05	-2.99				
60	0.02	0.04	-3.30				
60	0.04	0.05	-2.95				
60	0.03	0.04	-3.15				
90	0.02	0.04	-3.22				
90	0.04	0.05	-2.93				
90	0.02	0.04	-3.29				
120	0.01	0.03	-3.58				
120	0.01	0.03	-3.54				
120	0.02	0.04	-3.34				
20	0.02	0.04	-3.33				

The test values were inputted into the “Data Analysis-Regression” function in Microsoft Excel © where a summary output was generated. The summary output of the test values included the values for the x-variable (-4.4×10^{-3}) and

the standard error (S.E.) of the x-variable (8.7×10^{-3}). The procedure was repeated for the control values (x-variable = -0.15×10^{-3} , S.E. = 1.2×10^{-3}) and the degrees of freedom (df) was noted (total values – 2 = 28).

The formula for the student T-test is (equation 8):

$$T = \frac{x_{\text{test}} - x_{\text{ctrl}}}{[(SE_{\text{test}})^2 + (SE_{\text{ctrl}})^2]^{0.5}} \quad 8$$

The null hypothesis (H_0) is the slopes of the linear regression lines of the control and test data points are equivalent ($m_{\text{test}} = m_{\text{ctrl}}$; not significantly different where $\alpha = 0.05$) and the alternate hypothesis (H_A) is that the slopes are not equivalent ($m_{\text{test}} \neq m_{\text{ctrl}}$; significantly different at $\alpha = 0.05$). From a table of upper critical values of the Student's t-distribution (www.itl.nist.gov/div898/handbook/eda/section3/eda3672.htm), the T value at $\alpha = 0.05$, $df = 28$, $T_{0.05, 28} = 1.701$. The calculated value of $T_{\text{calc}} = 2.9$. Since the $T_{\text{calc}} > T_{0.05, 28}$, the slopes of the linear regression lines of the control and test data points for BaP in trial 1 are significantly different from each other. From T_{calc} , the p-value was calculated to be 7.2×10^{-3} (two-tailed) from: <http://www.danielsoper.com/statcalc/calc08.aspx>. Since the p-value calculated from T_{calc} is less than $p = 0.05$, that means that the slopes linear regression of the control and test data points are significantly different.

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