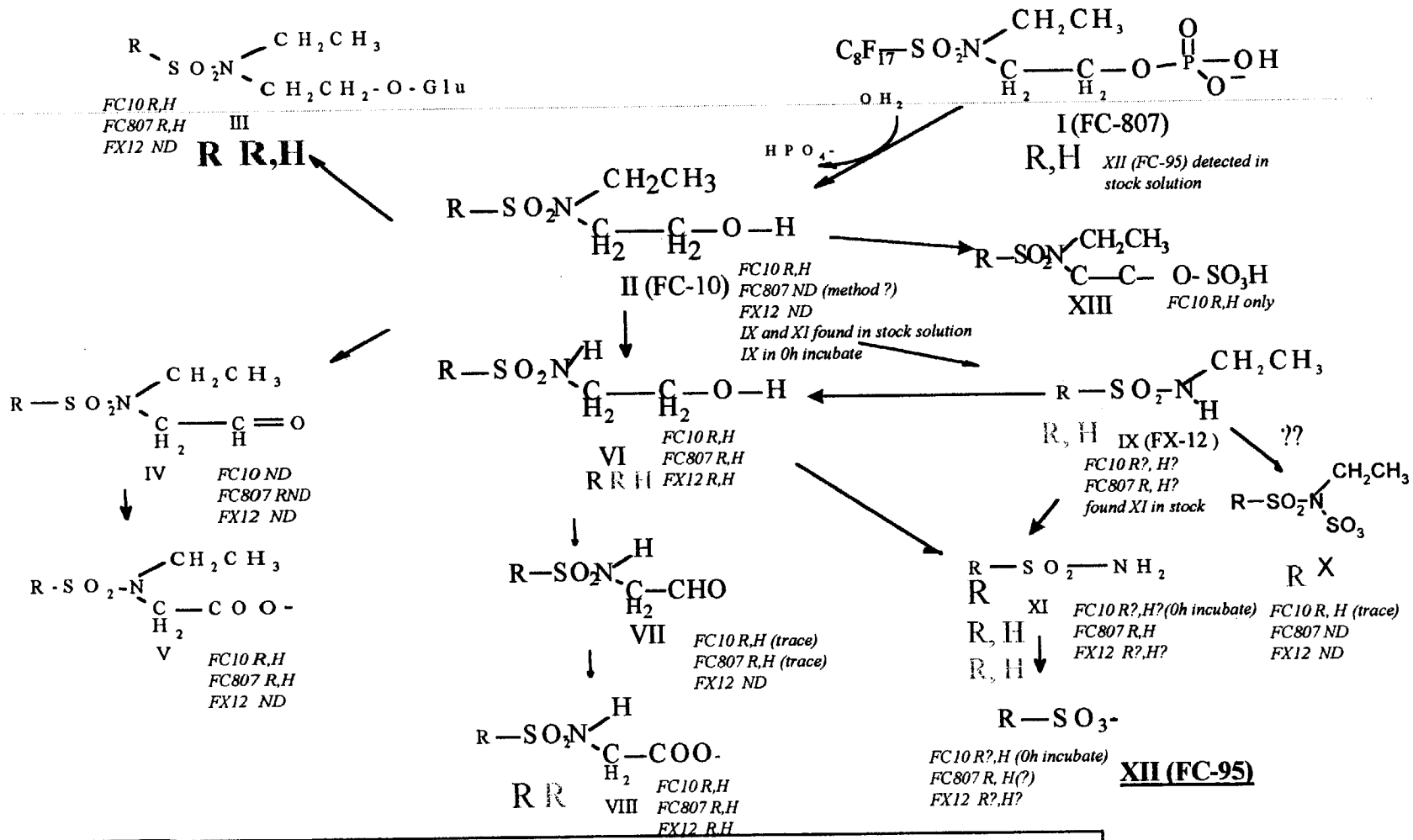
Perfluorosulfonamide Metabolism in Rat vs.. Human Hepatocytes*



i. FC-807
II. FC-10
IX. FX-12
XII FC-95
? compound found in stock or 0 hour incubate.

R	**Rat metabolites**
H	**Human metabolites**
Blue:	**Detected among metabolites of FC-807 (I)**
Green:	**Detected among metabolites of FC-10 (II)**
Red:	Detected among metabolites of FX-12 (IX)

Updated 2/5/98 based on 1/27/98 report from ABS using a more sensitive method (SRM) than previous draft reports.

3M MEDICAL DEPARTMENT, CORPORATE TOXICOLOGY

Protocol for Study Nos. T-6295.23; ST-46

Exploratory In-Vitro Percutaneous Absorption Study of Theophylline, Salicylic Acid, Perfluorooctylsulfonate, and Ammonium Perfluorooctanoate in SkinEthic Reconstituted Epidermis Model

***Study Objective*:**
This study is designed to gain familiarity with the SkinEthic Reconstituted Epidermis Model by conducting a simple absorption study on two previously characterized compounds (theophylline and salicylic acid in a paper by Doucet, et. al.), as well as one fluorochemical compound (perfluorooctylsulfonate, K^+). Supporting analytical work for this study will be done in the new Corporate Toxicology Laboratory in Building 236 as part of method and instrument qualification. A MTT assay will be conducted at the end of the absorption study to determine if SkinEthic viability was affected by application of the test compounds.

Research Client:
3M Specialty Chemicals Division
3M Center, Building 236
Saint Paul, MN 55144

Sponsor:
3M Specialty Chemicals Division
3M Center, Building 236
Saint Paul, MN 55144

Study Location:
3M Strategic Toxicology Laboratory
3M Center, Building 270-3S-06 room SB314
Saint Paul, MN 55144

Study Director:
John Butenhoff, Ph.D.
Senior Laboratory Manager
3M Medical Dept. / Corporate Toxicology
3M Center, Building 220-2E-02
Saint Paul, MN 55144
Ph.: 651-733-1962 FAX: 651-733-1773

Study Toxicologist:
Kathy Thompson, M.P.H.
Senior Toxicologist
3M Medical Dept. / Corporate Toxicology
3M Center, Building 220-2E-02
Saint Paul, MN 55144
Ph.: 651-733-9578 FAX: 651-733-1773

Proposed Study Timeline

Start Date: Wednesday, April 12th, 2000
End Date: Friday, April 14th, 2000
Analytical Completion Date: TBD
Final Report Completion Date: TBD

Regulatory Compliance:
This study will be performed in the 3M Strategic Toxicology Laboratory under a defined protocol and classified as a "Class C Study" as explained in TOX SOP 0950, Strategic Toxicology Lab GLP Program Procedure.

Test Material:
The fluorochemical test material will be provided by Dan Hakes, Product Responsibility Liaison, 3M Chemicals Division. The drug test material will be supplied by Sigma.

Identification:

Names:
Perfluorooctylsulfonate, K^+ (PFOS, K^+ salt)
Theophylline (THE)
Salicylic Acid (SA)

Molecular Formulas:

$C_8F_{17}SO_3^-K^+$	(PFOS)
C7-H8-N4-O2	(THE)
C7-H6-O3	(SA)

Lot Number: Documentation will be kept on file by the Sponsor for PFOS (Lot 217) and by the Corporate Toxicology Analytical Laboratory for THE and SA.

Purity:
Documentation will be kept on file by the Sponsor for PFOS, and by the Corporate Toxicology Analytical Laboratory for THE and SA.

Stability:
Documentation will be kept on file with the Sponsor for PFOS, and by the Corporate Toxicology Analytical Laboratory for THE and SA.

Storage Conditions:
Upon receipt, test material will be stored tightly sealed at room temperature.

Characteristics:
Information on synthesis methods, composition or other characteristics that define the test material will be kept on file with the Sponsor for PFOS, and by the Corporate Toxicology Analytical Laboratory for THE and SA.

SkinEthic Preparation Procedure:

The SkinEthic Reconstituted Epidermis will be prepared immediately upon arrival as per the manufacturers instructions (see Attachment One). Absorption testing will begin on April 13, 2000. All handling of SkinEthic material will be done using aseptic techniques.

Materials:

- Twelve SkinEthic Human Reconstituted Epidermis, Size 0.63 cm^2, and twelve blank filters
- SkinEthic Maintenance Medium (supplied by manufacturer)
- Additional empty 12 well culture plates (Becton Dickinson)
- Heating Block (37°C)
- Incubator (37°C, 5%CO_2, saturated humidity)

Reagents:

- Receptor Medium - Phosphate Buffered Saline (GibcoBRL Cat #14200-075, Lot # 1027781), 1.5% Bovine Serum Albumin (Sigma, A-8022 Lot#39H0691) added for FC compound
- Compound application vehicle – Milli-Q Water
- 1% Soap Solution (Tween 20, Mallinckrodt, lot #H285 N13H32)
- Trypsin (Sigma, lot #39H0952, cat#T-7418)
- MTT (Sigma, lot #39H5076, cat #M-2128)

Procedure:

- Unpack and maintain Skinethic Reconstituted Epidermis (REp) skin cell cultures and blank filter wells according to manufacturer's instructions in controlled temp and humidity incubator.

- Before product application, expose REp and blank filter wells to standard room environmental conditions for 2 hours to equilibrate with ambient atmosphere.

- Place 0.6 ml receptor medium in each well of 12-well culture plates.

- Transfer REp and blank filters gently with forceps to each well and place on heating block.

- Evenly apply 200 µl of THE and SA compound solutions to REp and blank filter surface in triplicate, 200 µl of PFOS compound solution in quadruplicate, and 200 µl of blank application vehicle (water) to the remainder and start timer:
  1. THE applied as saturated water solution of 6 mg/ml
  2. SA applied as saturated water solution of 2 mg/ml
  3. PFOS applied as a solution of 0.025 mg/ml (50 µM)

- Transfer REp and blank filter gently with forceps to new well containing receptor medium after 0.25 hr, 0.5 hr, 1 hr, 2 hr, 4 hr, and 6 hr.

- At end of experiment, place the receptor medium of each plate well into a polypropylene vial for later analysis.

- Wash the REp and blank filter surface 3 times with 200 µL of 1% soap solution, and rinse 3 times with same volume of water. Gently wipe cleaned surface with a cotton tip. Save washing fluids and tips in a polypropylene vial for later analysis.

- Conduct MTT Reduction assay on one sample for each test compound and two samples for negative control (water application only) to determine SE viability.

- Cut remaining skin membranes and blank filters into small pieces with scissors and digest for 48 hours with Trypsin at 40°C. After homogenization and centrifugation (1000 g) for 10 min, filter supernatant through a 0.45 µm filter and save in a polypropylene vial for later analysis.

Number of analytical samples generated : total =182:

Surface Washing Fluids:

A. PFOS: 8 at t = 6 hrs
B. THE: 6 at t = 6 hrs
C. SA: 6 at t = 6 hrs
D. Water: 4 at t = 6 hrs

Receptor Medium:

E. PFOS: 8 each at t = 0.25 hrs, 0.5 hrs, 1.0 hrs, 2.0 hrs, 4.0 hrs, 6.0 hrs
F. THE: 6 each at t = 0.25 hrs, 0.5 hrs, 1.0 hrs, 2.0 hrs, 4.0 hrs, 6.0 hrs
G. SA: 6 each at t = 0.25 hrs, 0.5 hrs, 1.0 hrs, 2.0 hrs, 4.0 hrs, 6.0 hrs
H. Water: 4 each at t = 0.25 hrs, 0.5 hrs, 1.0 hrs, 2.0 hrs, 4.0 hrs, 6.0 hrs

Digest Filtered Supernatant:

I. PFOS: 6 at t = 6 hrs
J. THE: 4 at t = 6 hrs
K. SA: 4 at t = 6 hrs

Ship samples on dry ice to:

Dave Ehresman, BS
Toxicology Specialist
3M Medical Dept. / Corporate Toxicology
3M Center, **Building 236-C146**
Saint Paul, MN 55144
Ph: 651-736-8410

for analysis of the test compound and the presumed metabolites (3-methylxanthine, 1,3,7-trimethyluric acid, and 1,3-dimethyluric acid for THE, gentisic acid for SA, none for PFOS).

Data Analysis:

Analytical results will be used to derive maximal flux rate in $\mu g/hour/cm^2$. The flux rate obtained for THE and SA will be compared to that reported in the paper by Doucet, et. al. Mass balance recovery should be $90 \pm 10\%$. MTT results will be used to determine if skin viability was affected (as compared to negative control) by application of the test compounds.

Responsibilities:

- Kathy Thompson will be responsible for performing the experiment and sending specimens for analysis.
- Dave Ehresman will be responsible for analytical evaluation of the samples.
- Kathy Thompson will draft a final report and ensure the report receives appropriate 3M review before a final report is issued, to be signed by Kathy Thompson and Dave Ehresman.

References:

Doucet, O., N. Garcia and L. Zastrow. Skin Culture Model: a Possible Alternative to the Use of Excised Human Skin for Assessing *In Vitro* Percutaneous Absorption. *Toxicology in Vitro* 12 (1998) 423-430.

Signatures:

John Butenhoff, Ph.D. Date
Senior Laboratory Manager
Study Director

Kathy Thompson, M.P.H. Date
Senior Toxicologist
Study Toxicologist

Sponsor Representative Date

3M MEDICAL DEPARTMENT, CORPORATE TOXICOLOGY

- ST-43: Standard Procedure for Liver Subcelular Fractionation -

Study Objective:

Perfluorochemical compounds such as perfluorooctane sulfonate (PFOS) have been linked with reduction in serum cholesterol levels and liver has been identified as the primary target site. The exact cellular mechanism of how perfluorochemical compounds interact with liver tissues is unknown and it is necessary to look into the sub-cellular fractions.

The purpose of this study is therefore to (1) isolate liver sub-cellular fractions and (2) to quantitate the PFOS contents in each fraction. The correlation between sub-cellular PFOS contents and serum biochemical markers will be further investigated.

This protocol is part of an ongoing study in the 3M Strategic Toxicology Laboratory designed to understand the effects of perfluorooctanesulfonamides and their metabolites, including PFOS, on intracellular fatty acid transport and metabolism, mitochondrial function, and cholesterol synthesis. This protocol provides a means of combining our resources and focus analytical capabilities on the same set of tissues.

Study Location:
3M Strategic Toxicology Laboratory
3M Center, Building 270-SB-314
Saint Paul, MN 55144-1000

Sponsor:
3M Specialty Chemicals
3M Center, Building 236
Saint Paul MN 55144-1000

Study Director:
Andrew M. Seacat Ph.D.
3M Medical Dept. Corporate Toxicology
3M Center Building 220-2E-02
Saint Paul, MN 55144-1000
Ph.: 651-575-3161, FAX: 651-733-1773

Study Toxicologist:
Sue Chang, M.S.
Advanced Research Toxicologist
M Medical Dept. / Corporate Toxicology
3M Center Building 220-2E-02
Saint Paul, MN 55144-1000
Ph.: 651-736-2212, FAX: 651-733-1773

Methods & Materials:

Regulatory Compliance:
This is an exploratory in nature and thus classified as non-GLP as explained in TOX SOP 0950, Strategic Toxicology Lab GLP Program Procedure.

Test Material:
The sponsor has provided samples of PFOS to the investigators. Analytical documentation of the starting material will be the responsibility of the sponsor. A chemical composition specification sheet will be kept on file.

Animals:

Species: Rat
Strain: Sprague Dawley
Source: Harlan Laboratories, Inc.
Age at initiation of treatment: 10-12 weeks old
Weight at initiation of treatment: approximately 250-300g

Animal Handling Cares and Specimen Collection:

Please see Study No. DT-15

Specimen Handling / Processing:

For perfluorosulfonamide metabolite analysis, liver samples will be fractionized according to the procedures described below. The isolated sub-cellular fractions will be packed in dry ice and shipped to Dr. Kris Hansen at 3M Environmental Lab.

Kris Hansen, Ph.D.
3M Environmental Technology and Safety Services
935 Bush Avenue
St. Paul, MN 55133-3331
Ph.: 651-778-6081, Fax: 651-778-6176.

Procedures:

Buffer needed:	0.25M sucrose in 0.05M Tris-HCl, pH 7.4
Equipment needed:	Tissue grinder Centrifuge Polypropylene centrifuge tubes (1.5 ml and 50 ml)
Animal:	Rat livers from both control and PFOS-treated groups.

(NOTE - All procedures should be carried out at 0 - 4°C.)

1. Suspend and homogenize the liver in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) using 20% weight/volume ratio, or approximately 5 volumes of the liver weights. The homogenization should be carried out on ice at all time to prevent the enzymes and tissue proteins from degradation.

   Homogenize on ice at low speed using a 50 ml Tenebrock tissue grinder with the hollow handle of the pestle filled with crushed ice. Homogenize at least 10 strokes, or until the suspension is visibly homogeneous.

   Record the total volume and the corresponding weights of the homogenates. Obtain two 1.5 ml microcentrifuge tubes and label them "1". Aliquot 1 ml of the homogenate into each tube. Store the tubes frozen for further analysis.

   Transfer the remaining of the homogenates into two 50-ml centrifuge tubes.

   Note: ***For this step and steps below following the centrifugation, make sure to measure and record the volumes and weights for each supernatant fraction.***

2. Spin the whole homogenate at 700 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "2". Store frozen.

   Pellet: The resulting pellet contains debris and unbroken cells. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**Plasma membrane**". Make two 1 ml aliquot of the suspension into two 1.5 ml microcentrifuge tubes labeled "3". Store frozen.

3. Spin the supernatants at 2500 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "4". Store frozen.

   Pellet: The resulting pellet contains nuclei and unbroken cells. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4). Label it as

"**nuclei**". Make two 1 ml aliquot of the suspension into two 1.5 ml microcentrifuge tubes labeled "**5**". Store frozen.

4. Centrifuge the supernatant fluid obtained from step 3 at 10,000 x g for 15 minutes at 4°C.

   Supernatant: Decant supernatant fluid into a new centirfuge tube. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**6**". Store frozen.

   Pellet: The resulting pellet contains mitochondria. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4). Label it as "**mitochondria**". Make two 1 ml aliquot of the mitochondrial suspension into two 1.5 ml microcentrifuge tubes labeled "**7**". Store frozen.

5. Centrifuge the supernatant fluid obtained from step 4 at 30,000 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**8**". Store frozen.

   Pellet: The resulting pellet contains lysosomes. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**lysosome**". Make two 1 ml aliquot of the lysosome suspension into two 1.5 ml microcentrifuge tubes labeled "**9**". Store frozen.

6. Centrifuge the supernatant fluid obtained from step 5 above at 105,000 x g for 60 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Label the supernatant as "**cytosol**". Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**10**". Store frozen.

   Pellet: The resulting pellet contains microsomes. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**microsome**". Make two 1 ml aliquot of the microsomal suspension into two 1.5 ml microcentrifuge tubes labeled "**11**". Store frozen.

Data:

- 3M Study Number = _____
- Date = _____
- Animal #, sex, and strain = _____
- Dose group = _____
- Weight of liver sample (g) = _____
- Volume of sucrose-Tris HCl added (mL) = _____

Fraction #		Fraction weight before aliquot (g)	*(Minus)* amount saved for LC-MS (mL)	*(Minus)* amout saved for gel electr. (mL)	Note
Whole homogenate					
Plasma membrane sup.	700 x g sup				
Nuclei sup.	2500 x g sup				
Mitochondrial sup.	10K x g sup				
Lysosomal sup.	30K x g sup				
Cytosol	105K x g sup				

Responsibilities:

Andrew Seacat and Sue Chang will be responsible for performing the experiment and sending specimens for analysis.

Signatures:

Andrew M. Seacat, Ph.D.
Toxicology Specialist
Study Director

Date

Sue Chang, M.S.
Advanced Research Toxicologist
Study Toxicologist

Date

Sponsor Representative

Date

Perfluorooctane Sulfonic Acid Induced HMG-CoA Reductase Inhibition in Pregnant Rats and Rat Pups.

Deanna J. Luebker, M.S., Advanced Research Toxicologist
Corporate Toxicology, 3M Medical Department
3M Center, Building 220-2E-02, Saint Paul, MN 55144
Phone (651) 737-1373 FAX (651) 736-2285
Djluebker@mmm.com

PREFACE

This project is designed to serve both as a 3M research project and as a project to fulfill the requirements of a Ph.D. thesis in Toxicology through the University of Minnesota Twin Cities. This proposal is being submitted with the understanding that publication of the results will be subject to a first-right-of-review by 3M.

PROPOSED PROJECT AND ITS PURPOSE

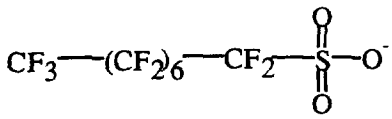
The objective of this study is to gain knowledge into the mechanism of action by which perfluorooctane sulfonic acid (PFOS) induces adverse effects on peri-/postnatal development in rats. The hypothesis that PFOS acts via inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol synthesis, will be examined. An attempt will be made to prevent PFOS toxicity by co-administrating mevalonate, the immediate product of HMG-CoA reductase. HMG-CoA reductase activity will be compared between dams and their pups and to age matched positive and negative controls. Assays will be performed to determine if PFOS acts directly on HMG-CoA reductase, indirectly via activation of AMP-activated protein kinase (AMP-PK) or through inducing an increase in AMP levels. Other parameters, which will be followed where possible, include body weight, liver weight, liver and serum PFOS, liver and serum lipids and miocardial fiber injury and/or inflammation.

INTRODUCTION

Perfluorooctane sulfonic acid (PFOS) is the ultimate metabolite of perfluoroalkyl acids and their derivatives, a class of chemicals produced by 3M and broadly utilized across multiple markets. The large production volume and broad-based utilization of this class of chemicals justifies the need for a comprehensive understanding of the potential adverse health effects they may pose. Because PFOS is the breakdown product of these chemicals, research focusing on its effects and mechanism(s) of action is of primary

concern. This proposal is aimed at gaining knowledge into the mechanism(s) by which PFOS induces effects on peri-/postnatal development in rats.

Figure 1 : Chemical Structure of PFOS



General effects seen in 3M PFOS reproduction studies in rats are reduced maternal body weight gain, increased percent stillborn and decreased pup survival (Case, presentation, 1999). The primary target organ identified from adult rat studies is the liver. Effects of PFOS treatment in adult rats include decreased body weight gain, increased liver weight, decreased serum cholesterol, triglycerides and bilirubin and mild peroxisome proliferation. The first detectable biological effect seen in adult rat PFOS studies is reduced serum cholesterol. At high doses of PFOS in rats, toxicity response curves become very steep and an apparent wasting syndrome occurs which culminates in death (3M Corporate Toxicology, unpublished, 1999).

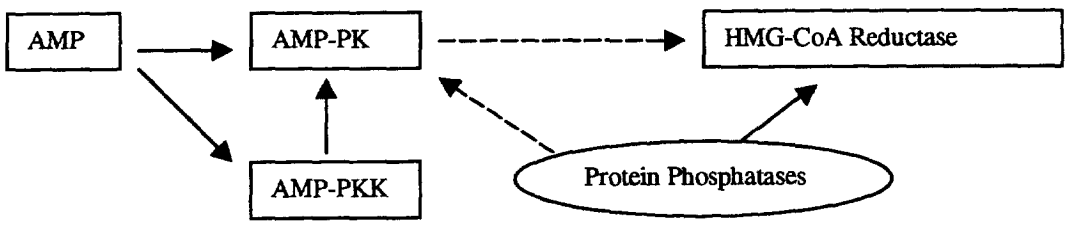
Haughom and Spydevold (1992) investigated the mechanism underlying the hypolipemic effect of PFOS in rats. They found PFOS to down regulate 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) activity to 35% of control. HMG-CoA reductase is a microsomal enzyme which controls the rate-limiting step in cholesterol synthesis, the conversion of HMG-CoA to mevalonate (HMG-CoA + 2 NADPH + 2 H^+ → mevalonate + 2$NADPH^+$ + CoA). The amount of HMG-CoA reductase present in a cell is controlled via negative feedback regulation through repressed gene transcription (Edwards *et al.*, 1983), inhibition of mRNA translation (Nakanishi *et al.*, 1988) and stimulation of the rate of degradation (Gill *et al.*, 1985). The primary mechanism by which this enzyme is controlled acutely is reversible phosphorylation (Gibson, 1985; Beg *et al.*, 1987; Hardie and Carling, 1997) with phosphorylation inactivating the enzyme. Other control mechanisms include reversible thiol-disulfide formation (Roitelman and Shechter, 1984a), allosteric activation by NAD(P)H (Roitelman and Shechter, 1984b) and alterations in membrane fluidity (Mitropoulos and Venkatesan, 1985).

AMP-activated protein kinase (AMP-PK) is the major HMG-CoA reductase kinase in rat liver (Carling *et al.*, 1989). It is controlled by reversible phosphorylation, with phosphorylation activating the kinase. AMP-activate protein kinase kinase (AMP-PKK) is the upstream component in this highly conserved kinase cascade (Hardie and Carling, 1997; Hardie, Carling and Carlson, 1998). Elevation of AMP can activate AMP-PK by allosterically activating AMP-PKK, by binding to AMP-PK and making it a poorer substrate for protein phosphatases, by binding to AMP-PK and making it a better substrate for the AMP-PKK and by allosterically activating AMP-PK (Corton *et al.*, 1995; Hawley *et al.*, 1995; Davies *et al.*, 1995). The AMP-PK cascade is therefore activated under conditions where AMP is elevated and ATP is depressed. The

consequences of AMP-PK activation include inactivation of ATP-consuming anabolic pathways (e.g., fatty acid synthesis via phosphorylation of acetyl CoA carboxylase) and sterol synthesis, via phosphorylation of HMG-CoA reductase (Corton *et al.*, 1995; Henin *et al.*, 1995). This cascade also activates the ATP-generating catabolic pathway of fatty acid oxidation via depression of malonyl-CoA levels (Merrill *et al.*, 1997; Hayashi *et al.*, 1998). AMP-PK could thus act to balance the supply of ATP to the demand of the nucleotide (Hardie *et al.*, 1999). This may be important during periods of cellular stress associated with ATP depletion, such as treatment with metabolic poisons in the hepatocyte (Corton, Gillespie and Hardie, 1994).

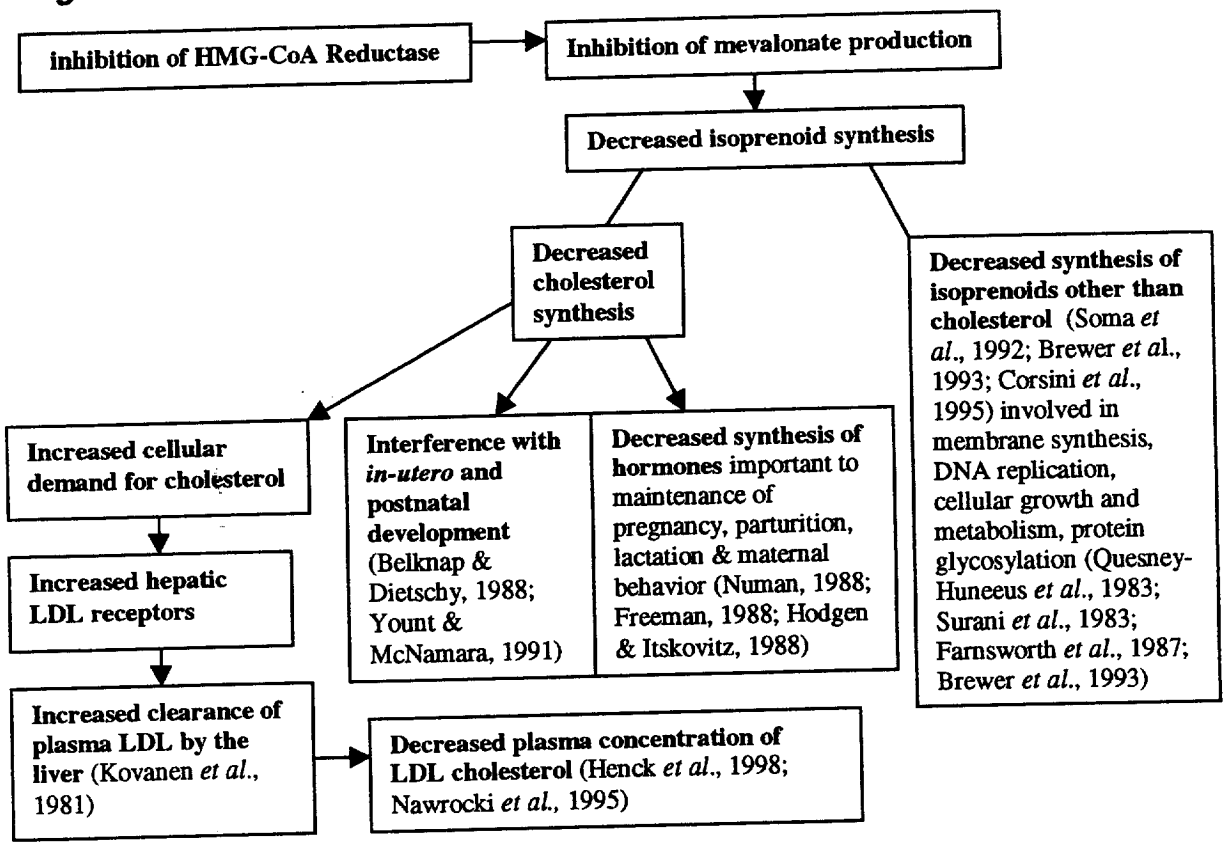
Figure 2: Reversible Phosphorylation of HMG-CoA Reductase

Solid line = activation, dotted line = inactivation



Inhibition of HMG-CoA reductase activity increases the cellular demand for cholesterol. This results in an increase in hepatic low density lipoprotein (LDL) receptors and an increase in clearance of plasma LDL by the liver (Kovanen *et al.*, 1981). Because LDL carries most of the cholesterol in humans, inhibition of HMG-CoA reductase results in a decrease in plasma cholesterol. Cholesterol, however, is an important precursor to hormones needed for the maintenance of pregnancy, parturition, lactation and maternal behavior (Numan, 1988; Freeman, 1988; Hodgen & Itskovitz, 1988) and is required for normal *in utero* and postnatal development in mammals (Belknap & Dietschy, 1988; Yount & McNamara, 1991). Inhibition of HMG-CoA reductase also leads to a deficiency of mevalonate and isoprenoids such as dolichol, isopentyl adenine, ubiquinone, haem A and many proteins bound to farnesyl and geranylgeranyl residues (Soma *et al.*, 1992; Brewer *et al.*, 1993; Corsini *et al.*, 1995). These isoprenoids are involved in membrane synthesis, DNA replication, cellular growth and metabolism and protein glycosylation (Quesney-Huneeus *et al.*, 1983; Surani *et al.*, 1983; Farnsworth *et al.*, 1987; Brewer *et al.*, 1993).

Figure 3: HMG-CoA Reductase Inhibition

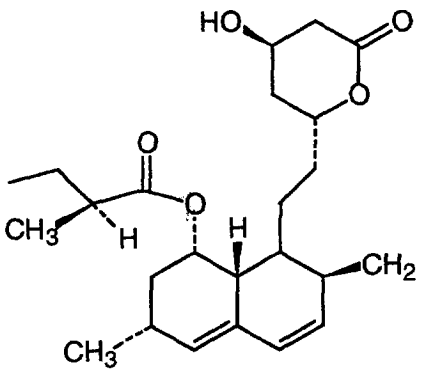


Statins, a class of compounds used to treat hyperlipidemia, act as competitive inhibitors of HMG-CoA reductase (Stryer, 1995). Some statins, for example lovastatin (Mevinolin, MEVACOR) and simvastatin (synvinolin, ZOCOR), exist as inactive "prodrugs" which must be enzymatically or chemically opened to their respective carboxylate forms to elicit inhibitory activity against HMG-CoA reductase (Corsini *et al.*, 1995). Other statins, for example pravastatin (eptastatin) and fluvastatin (LESCOL), are dosed in their active, open ring form (Corsini *et al.*, 1995). The active open-acid structures resemble that of HMG-CoA, the normal substrate for the reductase. Statins bind to the reductase, prevent it from binding to HMG-CoA and thus block the production of mevalonate.

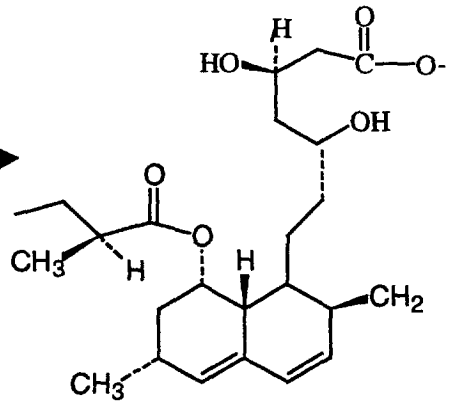
Figure 4 : Chemical Structures – Statins & HMG-CoA

Example Statins:

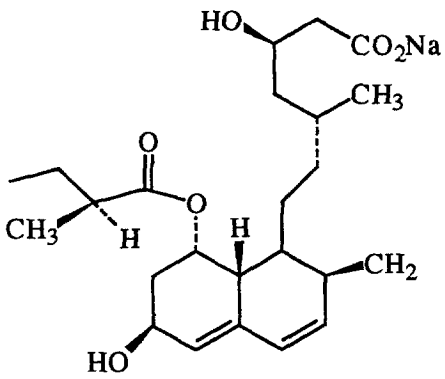
Lovastatin (Mevinolin, MEVACOR)
Inactive Prodrug



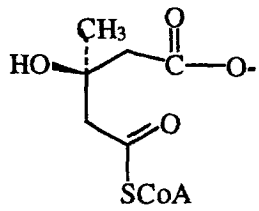
Mevinolinic Acid
Active Inhibitor



Pravastatin
Active Inhibitor



HMG-CoA:
Normal HMG-CoA reductase substrate

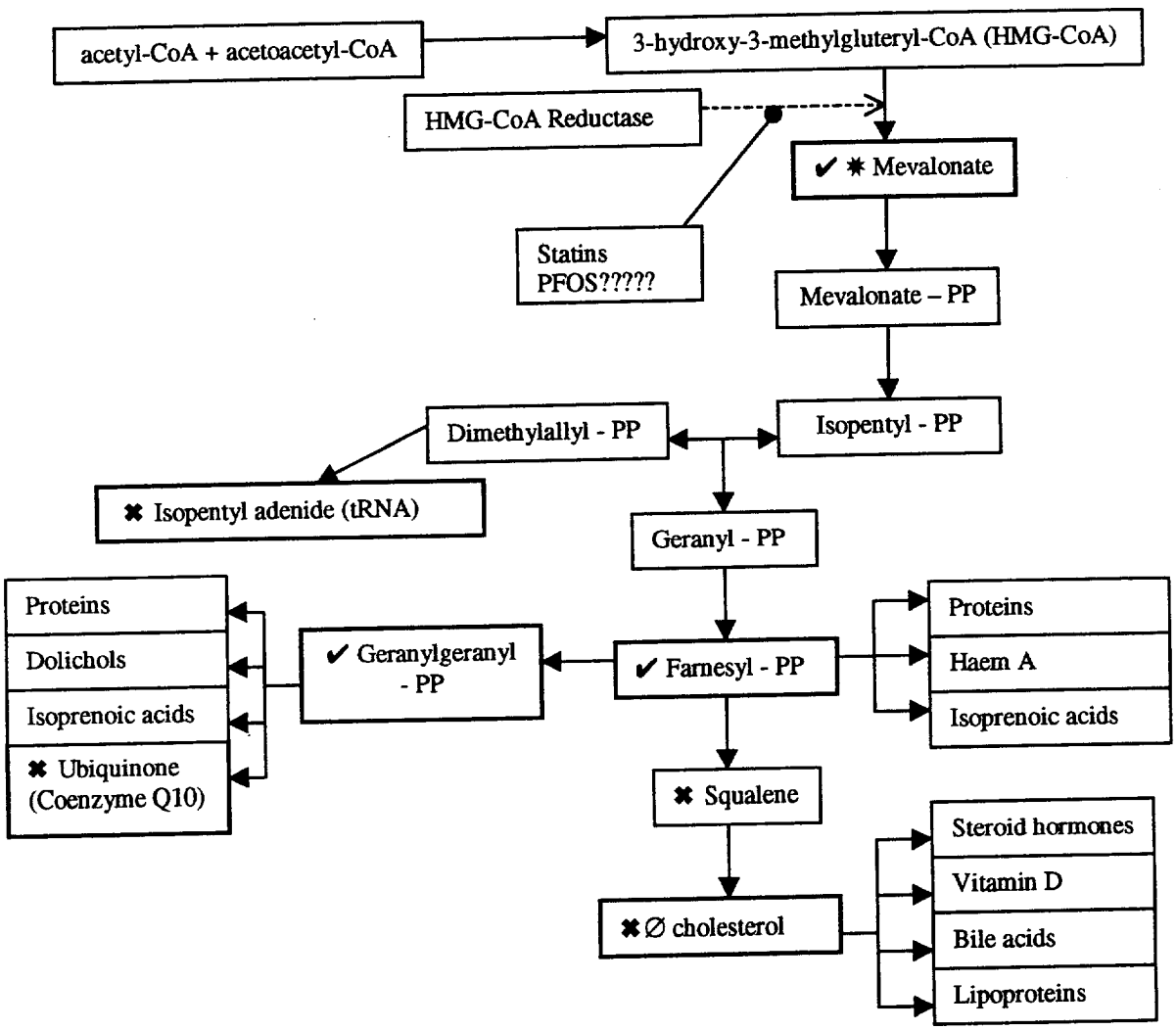


The majority of statins are known to be developmentally toxic to rats (Henck *et al.*, 1998). Effects include increased malformations (Minsker *et al.*, 1983); decreased post-weaning survival and delayed development of reflexes and behavior (FDA, 1987); reduced fetal body weight (Wise *et al.*, 1990a); reduced fetal and offspring body weights (Wise *et al.*, 1990b); reduced offspring body weight and increased swim maze errors (Minsker *et al.*, 1990); reduced offspring body weight and decrease neonatal survival (Hrab *et al.*, 1994); and retarded skeletal development and reduced fetal body weights (FDA, 1993). Studies have found that co-administration of mevalonate, the immediate product of HMG-CoA reductase, prevents or antagonizes various organ toxicities resulting from HMG-CoA reductase inhibition (MacDonald *et al.*, 1988; Kornbrust *et al.*, 1989; Minsker *et al.*, 1983; Hrab *et al.*, 1994). Minsker *et al.* (1983) found that co-administration of cholesterol, however, had no effect on the teratogenicity of lovastatin (Mevinolin, MEVACOR). Some statins are also known to produce myocardial effects.

Hrab *et al.* (1994) found significant cardiac myopathy in pregnant rats dosed with 24mg/kg/dy of fluvastatin (LESCOL). All animals dying in this dose group had cardiac lesions which were considered treatment related and believed to be associated to the cause of death (Hrab *et al.*, 1994). The authors concluded that inhibition of mevalonate and cholesterol synthesis was a significant factor contributing to the myocardial damage and/or death of the fluvastatin treated rats. Co-administration of mevalonate complete blocked and/or ameliorated the mortality, cardiac myopathy and other adverse effects seen with fluvastatin (Hrab *et al.*, 1994). *In-vitro* studies by Guijarro *et al.* (1998, 1999) found the lipophilic statins atorvastatin (LIPITOR), simvastatin (synvinolin, ZOCOR) and lovastatin (mevinolin, MEVACOR) to produce apoptosis of rat and human vascular smooth muscle cells (VSMC). Mevalonate administration completely prevented this apoptosis (Guijarro *et al.*, 1998, 1999). Several mevalonate metabolites were evaluated to determine which were involved in the statin-induced apoptosis. Cholesterol, isopentenyl adenine, ubiquinone (coenzyme Q10) and squalene failed to prevent the statin induced effects while the isoprenoids farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) fully reversed the effects (Guijarro *et al.*, 1998, 1999). These studies suggest the adverse effects seen with HMG-CoA reductase inhibitors may be more a result of decreased synthesis of certain isoprenoids than of decrease cholesterol synthesis.

Figure 5: Isoprenoid Biosynthetic Pathway

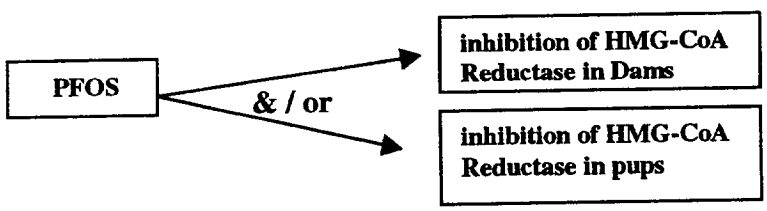
Key:
PP = pyrophosphate
✔ = shown to prevent statin induced VSMC apoptosis *in-vitro*[1]
✖ = shown NOT to prevent statin induced VSMC apoptosis *in-vitro*[1]
✷ = shown to prevent other statin induced adverse effects *in-vivo* and/or *in-vitro*[2]
∅ = shown NOT to prevent other statin induced adverse effects *in-vivo* and/or *in-vitro*[2]
[1] Guijarro *et al.*,1998 & 1999
[2] Minsker *et al.*, 1983; Surani *et al.*, 1983; Carson & Lennarz, 1979: Hrab *et al.*, 1994 MacDonald *et al.*, 1988; Kornbrust *et al.*, 1989, etc...)



Smith, Lear and Erickson (1995) found no sex related differences in HMG-CoA reductase enzyme activity in rats less than or equal to 28 days old. However, significantly higher activity levels are found in female rats as compared to males following puberty (Carlson, Mitchell and Goldfarb, 1978). Levin *et al.* (1989) investigated the developmental changes in expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers. Levels of HMG-CoA reductase mRNA undergo large fluctuations during rat liver ontogeny (McNamara, Quackenbush and Rodwell, 1972; Leoni *et al.*, 1984), being high prior to birth, declining to low levels during suckling (days 1-13 postnatal) and transiently increasing at weaning (day 19-21 postnatal). In humans, however, these mRNAs appear at an earlier phase of fetal life (by week 8) and undergo only minimal (< 3 fold) changes in concentration during the rest of the intrauterine as well as postnatal life (Levin *et al.*, 1989). Fetal and early postnatal rats may thus be very sensitive to inhibitors of HMG-CoA reductase activity while fetal and early postnatal humans may not.

The study outlined in this proposal is designed to help determine how PFOS administration in pregnant rats results in reduced maternal body weight gain, increased percent stillborn pups and decreased pup survival after birth. The hypothesis to be tested is that PFOS acts to inhibit HMG-CoA reductase activity in pregnant rats and/or their offspring and thus can behave as a developmental toxicant at higher dose levels. The results of this study, along with what is already known about human HMG-CoA reductase activity, will help determine the potential risk PFOS holds to be a developmental toxicant in humans.

Figure 6: Hypothesis



RESEARCH PLAN

OBJECTIVES

1. Determine whether hepatic HMG-CoA reductase activity is inhibited in PFOS treated pregnant rats.

2. Determine whether hepatic HMG-CoA reductase activity is inhibited in rat pups from PFOS treated dams.

3. Investigate the mechanism by which PFOS acts to inhibit HMG-CoA reductase activity.

   a) Determine if PFOS acts directly on HMG-CoA reductase.
   b) Determine if PFOS acts on AMP-PK.
   c) Determine if PFOS induces an increase in cellular AMP levels.

EXPERIMENTAL APPROACH

All results will be compared between dams and their pups to identify any relationship between dam effect and pup effect. Body weight and total liver weight will be recorded for each dam and litter as indices of general health and hepatomegally. Each group of dams and pups will be compared to age matched positive controls (dosed with a statin), negative controls (dosed with vehicle) and mevalonate challenged controls. Other parameters that will be followed where possible include liver and serum PFOS levels, liver and serum lipid levels and myocardial fiber injury and inflammation. As deemed necessary, portions of the study may be sent to contract labs for completion.

Table 1: Dose Groups

	Group	Compound(s)
PFOS Groups	1	PFOS
	2	PFOS + mevalonate
	3	PFOS vehicle control
Positive Control Groups	4	statin
	5	statin + mevalonate
	6	statin vehicle control

In-Vitro Phase

HMG-CoA reductase

The ability of PFOS to directly inhibit HMG-CoA reductase will be examined using rat liver microsomes. Microsomes will be prepared from untreated female rats with and without sodium fluoride (NaF) in all buffers and assay media. NaF inhibits phosphoprotein phosphatase activity (Kato and Bishop, 1972) and thus, inclusion of NaF at all stages of isolation and purification severely inhibits the conversion of inactive (phosphorylated) reductase to the active (unphosphorylated) form (Nordstrom, Rodwell and Mitchelen, 1977). Microsomes will be dosed as listed in table 1 and, following an incubation period, assayed for total and active HMG-CoA reductase by omission and inclusion, respectively, of NaF. Activity will be calculated as the rate (picomoles/minute) of formation of [^{3}H]mevalonate from [^{3}H]HMG-CoA per milligram of microsomal protein. Lipid levels of the various dosed microsomes will be measured using commercially available kits and/or HPLC. Cholesterol production in cultured hepatocytes, a biomarker of HMG-CoA reductase activity, may be used as a secondary measure of *in-vitro* HMG-CoA reductase inhibition.

AMP levels

To determine whether PFOS induces an increase in cellular AMP *in-vitro*, AMP levels of the microsomes will be quantified using HPLC.

AMP-PK

To assess the ability of PFOS to act directly to activate AMP-PK *in-vitro*, a peptide assay developed by Davies, Carling and Hardie (1989) which uses a synthetic peptide substrate, SAMS peptide, will be used. SAMS peptide was developed based on the amino acid sequence (Ser79) which is phosphorylated exclusively by AMP-PK. AMP-PK specimens will be prepared by partially purifying the cytosolic protein fraction of control rat liver as described by Carling *et al.* (1989). These specimens will be treated as outlined in table 1. Following an incubation period, AMP-PK activity will be measured based on the incorporation of radioactivity from [γ-^{32}P]ATP into the SAMS peptide (Davies *et al.*, 1989).

In-Vivo Phase

HMG-CoA Reductase Activity in Pregnant Rats and Rat Pups

Isolation of cellular subfractions to directly evaluate HMG-CoA reductase activity results in dissociation of the drug from the enzyme, which returns catalytically active (Corsini *et al.*, 1995). The most favorable method to detect *in-vivo* inhibition of HMG-CoA reductase is by determining the *in-vivo* rate of incorporation of precursors, for example acetate, into cholesterol (Corsini *et al.*, 1995). The potency of PFOS to inhibit the conversion of ^{14}C-acetate into ^{14}C-cholesterol *in-vivo*, therefore, will be used as a biomarker to determine HMG-CoA reductase in dams. These results should parallel the inhibition of HMG-CoA reductase inhibition found in the *in-vitro* assays. Other biomarkers which have been used successfully include urinary and plasma mevalonate levels (Corsini *et al.*, 1995).

Pregnant rats will be repeat dosed during gestation according to the dose groups listed in table 1. Prior to euthanization, dams will be dosed with ^{14}C-acetate. Half of all dams will be allowed to come to term and half will be euthanized the morning of day 21 of gestation and pups will be removed. If PFOS is a HMG-CoA reductase inhibitor, the reduced maternal weight gain, increased stillborn and decreased pup survival seen in previous studies should be eliminated or ameliorated in the mevalonate supplemented dose group. Plasma and/or urine may be collected and analyzed for mevalonate to use as additional biomarkers of HMG-CoA reductase activity in the dams. Plasma mevalonate levels will serve as the biomarker to determine the degree of HMG-CoA reductase inhibition in each litter. Liver (from each dam and pooled for each litter) will be used for AMP-PK activity determination, liver lipid analysis and AMP determination. Sera will be collected from each dam and pooled for each litter for PFOS determination and serum

lipid analysis. Liver and serum high density lipoprotein (HDL), low density lipoprotein (LDL) and total cholesterol will be determined using commercially available kits and/or HPLC. Liver and serum PFOS determination will be performed by the 3M Environmental Laboratory Fluorine Analytical Chemistry Team (FACT) headed by Kris Hansen, Ph.D. Myocardial tissue will be examined by electron microscopy (EM) or other appropriate method for fiber injury and/or inflammation.

Liver AMP Levels in Pregnant Rats and Pups

To determine whether PFOS induces an increase in cellular AMP levels *in-vivo*, liver AMP levels will be determined using HPLC.

AMP-PK Activity in Pregnant Rats and Pups

AMP-PK specimens will be prepared by partially purifying the cytosolic protein fraction as described by Carling *et al.* (1989). AMP-PK activity will be measured based on the incorporation of radioactivity from [γ-^{32}P]ATP into the SAMS peptide (Davies *et al.*, 1989).

SUMMARY

The study outlined in this proposal is aimed at gaining knowledge into the mechanism(s) by which PFOS leads to reduced maternal body weight gain, increased percent stillborn and decreased pup survival in rats. The hypothesis that PFOS acts via inhibition of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, will be examined. PFOS has previously been shown to down regulate HMG-CoA reductase activity in rats (Haughom and Spydevold, 1992). Several HMG-CoA reductase inhibitors (statins) have been shown to induce developmental effects similar to those seen with PFOS (Minsker *et al.*, 1983; FDA, 1987; Wise *et al.*, 1990a; Wise *et al.*, 1990b; Minsker *et al.*, 1990; Hrab *et al.*, 1994; FDA, 1993). Studies have found that co-administration of mevalonate, the immediate product of HMG-CoA reductase, prevents or antagonizes various organ toxicities resulting from HMG-CoA reductase inhibition in rats and rabbits (MacDonald *et al.*, 1988; Kornbrust *et al.*, 1989; Minsker *et al.*, 1983; Hrab *et al.*, 1994). An attempt will be made to prevent PFOS toxicity by co-administrating mevalonate. HMG-CoA reductase activity will be compared between dams and their pups and to age matched positive and negative controls. Specimens will be examined to determine if PFOS acts directly on HMG-CoA reductase, indirectly via activation of AMP-activated protein kinase (AMP-PK) or through inducing an increase in AMP levels. Other parameters, which will be followed where possible, include body weight, liver weight, liver and serum PFOS, liver and serum lipids and myocardial fiber injury and inflammation. Due to normal developmental differences in HMG-CoA reductase activity, fetal and early postnatal rats may be much more sensitive to HMG-CoA reductase inhibition than are fetal and early postnatal humans (Levin *et al.*, 1989). The results of this study, taken with what is already known about HMG-CoA reductase activity, will help determine the mechanism by which high doses of PFOS cause adverse effects in pregnant rats and their pups and the potential risk PFOS holds to be a developmental toxicant in humans.

REFERENCES

Beg Z., Stonik J. and Brewer H. Jr. (1987). Modulation of the enzymatic activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by multiple kinase systems involving reversible phosphorylation: A review. *Metabolism.* **36(9)**: 900-917.

Belknap W. and Dietschy J. (1988). Sterol synthesis and low density lipoprotein clearance in vivo in the pregnant rat, placenta and fetus. *J. Clin. Invest.* **82**: 2077-2085.

Brewer L., Sheardown S. and Brown N. (1993). HMG-CoA reductase mRNA in the post-implantation rat embryo studied by in situ hybridization. *Teratology.* **47**: 137-146.

Carling D., Clarke P., Zammit V. and Hardie D. (1989). Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur J Biochem.* **86(1-2)**: 129-136.

Carlson S., Mitchell A. and Goldfarb S. (1978). Sex-related differences in diurnal activities and development of hepatic microsomal 3-hydroxy-3-methyl-glutaryl coenzyme A reductase and cholesterol 7α-hydroxylase. *Biochem. Biophys. Acta.* **531**: 115-124.

Carson D. and Lennarz W. (1979). Inhibition of polyisoprenoid and glycoprotein biosynthesis causes abnormal embryonic development. *Proc. Natl. Acad. Sci. USA.* **76**: 5709-5713.

Case M. (June 16, 1999). 3M Toxicology Presentation.

Corsini A., Maggi F. and Catapano A. (1995). Pharmacology of competitive inhibitors of HMG-CoA reductase. *Pharmacol. Res.* **31**: 9-27.

Corton J., Gillespie J., Hawley S. and Hardie D. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur. J. Biochem.* **229(2)**: 558-565.

Corton J., Gillespie J. and Hardie D. (1994). Role of the AMP-activated protein kinase in the cellular stress response. *Curr. Biol.* **4**: 315-324.

Davies S., Helps N., Cohen P. and Hardie D. (1995). 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett.* **377(3)**: 421-425.

Davies S., Carling D. and Hardie D. (1989). Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **186**: 123-128.

Edwards P., Lan S., Tanaka R. and Fogelman A. (1983). Mevalonolactone inhibits the rate of synthesis and enhances the rate of degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rat hepatocytes. *J. Biol. Chem.* **258(12):** 7272-7275.

Farnsworth W., Hoeg J., Maher M., Brittain E., Sherins R. and Brewer H. Jr. (1987). Testicular function in Type II hyperlipoproteinemic patients treated with lovastatin (mevinolin) or neomycin. *J. Clin. Endocrinol. Metab.* **65**: 546-550.

FDA. (1987). Summary basis of approval of NDA 19-643 (lovastatin), 1-28.

FDA. (1993). Summary basis of approval of NDA 20-261 (fluvastatin), 51-54 and amendment, pp. 1 & 2.

Freeman M. (1988). The ovarian cycle of the rat. pp. 1893-1928 In The Physiology of Reproduction, Vol 2. Ed. Knobil J. and Neill J., New York: Raven Press.

Gibson B. (1985). pp. 79-131 In Regulation of HMG-CoA Reductase. Ed. Preiss B., New York: Academic Press.

Gill J. Jr, Beach M. and Rodwell V. (1985). Mevalonate utilization in Pseudomonas sp. M. Purification and characterization of an inducible 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **260(16):** 9393-9398 .

Guijarro C., Blanco-Colio L., Ortego M., Alonso C., Ortiz A., Plaza J., Diaz C., Hernandez G. and Egido J. (1998). 3-hydroxy-3-methylgluteryl coenzyme A reductase and isoprenylation inhibitors induce apoptosis of vascular smooth muscle cells in culture. *Circ. Res.* **83**:490-500.

Guijarro C., Blanco-Colio L., Massy Z., O'Donnell M., Kasiske B., Keane W. and Egido J. (1999). Lipophilic statins induce apoptosis of human vascular smooth muscle cells. *Kidney Internal.* **57(71):** S88-S91.

Hardie D. and Carling D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur. J. Biochem.* **246(2)**: 259-273.

Hardie D., Salt I., Hawley S. and Davies S. (1999). AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. *Biochem. J.* **338**: 717-722.

Hardie D., Carling D. and Carlson M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **67**: 821-855.

Haughom B. and Spydevold θ. (1992). The mechanism underlying the hypolidemic effect of perfluorooctanic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and colfibric acid. *Biochim. Biophys. Acta.* **1128**: 65-72.

Hawley S., Selbert M., Goldstein E., Edelman A., Carling D. and Hardie D. (1995). 5'-AMP activates the AMP-activated protein kinase cascade, and Ca2+/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. *J. Biol. Chem.* **270(45)**: 27186-27191.

Hayashi T., Hirshman M., Kurth E., Winder W. and Goodyear L. (1998). Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes.* **47(8)**:1369-1373.

Henck J., Craft W., Black A., Colgin J. and Anderson J. (1998). Pre- and postnatal toxicity of the HMG-CoA reductase inhibitor atrovastatin in rats. *Toxicol. Sci.* **41**: 88-99.

Henin N., Vincent M., Gruber H. and Van den Berghe G. (1995). Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. *FASEB J.* **9(7)**: 541-546.

Hodgen G. and Itskovitz J. (1988). Recognition and maintenance of pregnancy. pp. 1995-2021 In The Physiology of Reproduction, Vol. 2. Ed. Knobil E. and Neill J. New York: Raven Press.

Hrab R., Hartman H. and Cox R. (1994). Prevention of fluvastatin-induced toxicity, mortality, and cardiac myopathy in pregnant rats by mevalonic acid supplementation. *Teratology*, **50**: 19-26.

Kato K. and Bishop J. (1972). Glycogen synthetase-D phosphatase. I. Some new properties of the partially purified enzyme from rabbit skeletal muscle. *J. Biol. Chem.* **247(22)**: 7420-7429.

Kornbrust D., MacDonald J., Peter C., Duchai D., Stubbs R., Germershausen J. and Alberts A. (1989). Toxicity of the HMG-Coenzyme A reductase inhibitor, lovastatin, to rabbits. *J. Pharmacol. Exp. Ther.* **248**: 498-505.

Kovanen P., Bilheimer D., Goldstein J., Jaramillo J. and Brown M. (1981). Regulatory role for hepatic low density lipoprotein receptors in vivo in the dog. *Proc. Natl. Acad. Sci. USA.* **78**:1194-1198.

Leoni S., Spagnuolo S., Conti-Devirgiliis L., Dini L., Mangiantini M. and Trentalance A. (1983). Cholesterogenesis and related enzymes in isolated rat hepatocytes during pre- and postnatal life. *J. Cell Physiol.* **118(1)**: 62-66.

Levin M., Pitt A., Schwartz A., Edwards P. and Gordon J. (1989). Developmental changes in expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers. *Biochim. Biophys. Acta.* **1003**: 293-300.

MacDonald J., Gerson R., Kornburst D., Kloss M., Prahalada S., Berry P., Alberts A. and Bokelman D. (1988). Preclinical evaluation of lovastatin. *Am. J. Cardiol.* **62**: 16-27.

McNamara D., Quackenbush F. and Rodwell V. (1972). Regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. Developmental Pattern. *J. Biol. Chem.* **247**: 5805-5810.

Merrill G., Kurth E., Hardie D. and Winder W. (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am. J. Physiol.* **273:** E1107-E1112.

Minsker D., MacDonald J., Robertson R. and Bokelman D. (1983). Mevalonate supplementation in pregnant rats suppresses the teratogenicity of mevinolinic acid, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Teratology*, **38**: 449-456.

Minsker D., Robertson R., Bokelman D., Akutsu S. and Fujii T. (1990). Simvastatin (MK-0733): Oral late gestation and early lactation study in rats. *Oyo Yakuri.* **39:** 169-179.

Mitropoulos K. and Venkatesan S. (1985). Membrane-mediated control of HMG-CoA reductase activite. In Regulation of HMG-CoA Reductase. Ed. Preiss B., Orlando: Academic Press Inc.

3M Corporate Toxicology. (1999).Unpublished Fluorochemical Studies.

Nakanishi M., Goldstein J. and Brown M. (1988). Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J. Biol. Chem.* **263(18):** 8929-8937.

Nordstrom J., Rodwell V. and Mitchelen J. (1977). Interconversion of active and inactive forms of rat liver hydroxymethylglutaryl-CoA reductase. *J. Biol. Chem.* **252 (24)**: 8924-8934.

Numan M. (1988). Maternal behavior. pp.1569-1645 In The Physiology of Reproduction, Vol. 2, Ed. Knobil E. and Neill J., New York: Raven Press.

Quesney-Huneeus V., Galick H. and Siperstein M. (1983). The dual role of mevalonate in the cell cycle. *J. Biol. Chem.* **258**: 378-385.

Roitelman J. and Shechter I. (1984a). Regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Evidence for thiol-dependent allosteric modulation of enzyme activity. *J. Biol. Chem.* **259(2):** 870-877.

Roitelman J. and Shechter I. (1984b). Allosteric activation of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase by nicotinamide adenine dinucleotides. *J. Biol. Chem.* **259(22):** 14029-14032.

Smith J., Lear S. and Erickson S. (1995). Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J. Lipid Res.* **36**: 641-652.

Soma M., Corsini A. and Paoletti R. (1992). Cholesterol and mevalonic acid modulation in cell metabolism and multiplication. *Toxicol. Lett.* **64/65**: 1-15.

Stryer L. (1995). Biosynthesis of membrane lipids and steroids. Chapter 27 in Biochemistry - Fourth Edition. New York: W.H. Freeman and Company.

Surani M., Kimber S. and Osborn J. (1983). Mevalonate reverses the developmental arrest of preimplantation mouse embryos by Compactin, an inhibitor of HMG-CoA reductase. *J. Embryol. Exp. Morph.* **75**: 205-223.

Wise L., Majka J., Robertson R., et. al. (1990a). Simvastatin (MK-0733): Oral teratogenicity study in rats pre- and postnatal observations. *Oya Yakuri.* **39**: 143-158.

Wise L., Minsker D., Robertson R., Bokelman D., Akutsu S. and Fujii T. (1990b). Simvastatin (MK-0773): Oral fertility study in rats. *Oya Yakuri.* **39**: 127-141.

Yount N. and McNamara D. (1991). Dietary regulation of maternal and fetal cholesterol metabolism in the guinea pig. *Biochim. Biophys. Acta.* **1085**: 82-90.