AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON LIVER FATTY ACID-BINDING PROTEIN.

DEANNA J. NABBEFELD

Masters Thesis Key

FC1: Perfluorooctane Sulfonic Acid (PFOS)

FC2: Ammonium Perfluorooctonate (APFO)

FC3: N-ethylperfluorooctane Sulfonamide Ethanol (N-EtFOSE)

FC4: N-ethylperfluorooctane Sulfonamide (N-Et FOSE Amide; FX-12)

AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON LIVER FATTY ACID-BINDING PROTEIN.

A THESIS
SUBMITTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY
DEANNA J. NABBEFELD

IN FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE/ENVIRONMENTAL HEALTH

APRIL, 1998

AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON LIVER FATTY ACID-BINDING PROTEIN.

ABSTRACT

The objective of this study was to investigate the hypothesis that certain Fluorocarbons (FCs) bind liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event leading to peroxisome proliferation. The goals of the study were to assess the effect of FCs on L-FABP function as evaluated by the ability of the fluorescent FA analogue 11 - (5-dimethylaminonapthalenesulphonyl) - undecanoic acid (DAUDA) to bind to L-FABP isolated from rats and guinea pigs treated and not treated with FC1 *in vivo*; and to assess the potency of FC1, FC2, FC3 and FC4 for binding to L-FABP.

Results show a decreased maximum binding capacity of L-FABP from FC1 treated rats without an increase in Kd. The most potent L-FABP binder was FC1, followed by FC4 and (with equal IC_{50}s) FC3 and FC2. Results for guinea pig L-FABP samples were inconclusive. This may be because guinea pig samples were only partially purified; thus resulting in a high degree of interference from remaining cellular debris and a lower concentration of L-FABP, as a proportion of total protein, as compared to rat samples.

INTRODUCTION

STUDY OBJECTIVES

This study was designed to investigate the hypothesis that certain fluorocarbons (FCs) bind to liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event leading to peroxisome proliferation. To examine this hypothesis, the kinetics of FA and FC binding to L-FABP were investigated with an *in vitro* binding assay using the fluorescent FA analogue 11 - (5-dimethylaminonapthalenesulphonyl) - undecanoic acid (DAUDA). FC1 and FC2, known peroxisome proliferators, and FC3 and FC4, suspect peroxisome proliferators, were examined. Wyeth-14,643 (WY), a well known peroxisome proliferator, was used as the positive control and methanol as the negative control. Oleic acid, a FA known to bind to L-FABP with a very high affinity, was used to measure the maximum L-FABP binding. (Figure 1 - structures). L-FABP from

male rats (considered to be strong responders to peroxisome proliferators) and male guinea pigs (considered to be weak or non-responders to peroxisome proliferators) (Svoboda, Grady and Azarnoff, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986), treated or not treated with FC1 *in vivo,* were examined.

The goals of the study were as follows:

1) to assess the effect of FCs on L-FABP function, as evaluated by the ability of DAUDA to bind to L-FABP isolated from rats and guinea pigs; and

2) to assess the potency of the various FCs for binding to L-FABP.

The first goal was accompilished as follows:

a. L-FABP from rats and guinea pigs, treated and not treated with FC1, was isolated;

b. the maximum binding capacity or receptor number (Bmax) of each L-FABP sample and the dissociation constant or affinity (Kd) of DAUDA for each L-FABP sample were calculated; and

c. the concentration of oleic acid which inhibited 50% of specific DAUDA binding to isolated L-FABP samples, the oleic acid IC_{50} for each sample, was measured.

The second goal was achieved by calculating the IC_{50} of each FC for the binding of DAUDA to the isolated control rat L-FABP sample.

FIGURE 1 - STRUCTURES.

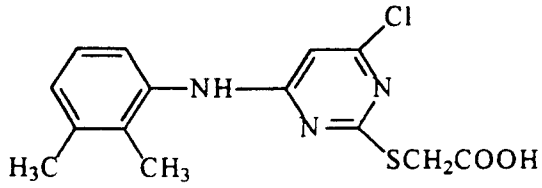
FC1

FC2

FC3

FC4

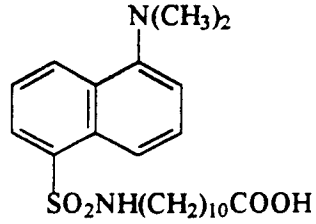
Wyeth-14,643



Methanol

$CH_3—OH$

DAUDA



Oleic Acid

$CH_3—(CH_2)_7—CH=CH—(CH_2)_7—COOH$

BACKGROUND

LIVER FATTY ACID-BINDING PROTEIN

The exact role of L-FABP, a member of the intracellular lipid-binding protein (iLBP) family, is unclear (Bass, Kaikaus and Ockner, 1993). It is found predominately in the liver, although it is also present in the small intestinal and colonic enterocytes, gastric brush border, and enteroendocrine cells (Bass, 1985; Bass, 1988; Sweetser, Heuckeroth and Gordon, 1987; Vincent and Muller-Eberhard, 1985; Chan *et al.*, 1985; Gordon *et al.*, 1982; Sorof and Custer, 1987). Accepted functions of L-FABP include binding and transporting FAs within the cell, regulating lipid metabolism, and protecting the cell by maintaining the concentration of free fatty acids (FFAs) below toxic levels (Bass *et al.*, 1993). L-FABP is unique to the iLBP family in that it has a larger binding cavity (Thompson *et al.*, 1997); broader ligand specificity (binds multiple hydrophobic compounds such as heme, certain eicosanoids, bilirubin, thyroxine, steroids, specific carcinogens and peroxisome proliferators as well as FAs) (Kaikaus, Bass and Ockner, 1990; Ockner *et al.*, 1972; Rolf *et al.*, 1995; Thumser, Voysey and Wilton, 1994; Khan and Sorof, 1990; Levi, Gatmaitan and Area, 1969); and the ability to bind two molecules per protein while other iLBPs bind only one (Thompson *et al.*, 1997).

The crystal structure of rat L-FABP (Thompson *et al*, 1997) reveals two short antiparallel α-helices positioned over one end of an 11-stranded antiparallel β-barrel. This differs from other iLBPs, which are 10-stranded, but does not significantly alter the conformation of the protein. A cavity is formed within the β-barrel that serves as an internalized ligand

binding site with polar and nonpolar residues and bound water. In addition to a normal gap between the two β-strands, L-FABP has a second gap formed by missing hydrogen bonds. The function of this gap is unknown, but the missing hydrogen bonds increase the range of motion in L-FABP compared to other iLBPs. This localized conformational flexibility may contribute to the broad ligand specificity exhibited by L-FABP.

Two L-FABP binding sites exist, and interact allosterically. Crystal structures of the protein prepared with oleic acid have characterized the primary binding site by an internalized carboxylate and a U-shaped hydrocarbon chain. Fatty acids bound in the primary binding site interact with Arg^{122}, a conserved residue in all iLBPs; and are surrounded by protein atoms, structural water and nearby atoms of the second bound FA. The oleic acid in the primary binding site is involved in hydrogen bond interactions at the carboxyl group with Ser^{39}, Arg^{122} and Ser^{124}. The secondary binding site is characterized by having the carboxylate of the second oleic acid near the surface, and the hydrocarbon tail inserted toward the center of the molecule and between the U-shaped hydrocarbon chain in the primary binding site. The carboxylate at this site is solvent-accessible, but still involved in a network of hydrogen bonds with residues forming the entrance to the primary binding cavity. The two ligands are in physical contact and it is believed that they influence each others relative affinities. Structural data suggest the second site may not exist until the primary site is filled, or that the prior presence of a FA in the primary site may be required for anything larger that a C_{14} FA to bind the secondary site (Thompson *et al.*, 1997).

FLUOROCARBONS

Fluorocarbons (FCs) are compounds structurally analogous to hydrocarbons with the hydrogens replaced with fluorines. The FCs under investigation resemble long chain FAs, having a hydrophobic tail and a polar head group. The tails of FCs are more rigid in structure than the tails of FAs, however, and thus the conformational flexibility of FCs is more restricted than that of FAs (Zisman, 1964). FCs have unique chemical and physical properties such as being very heat stable, inert and chemically and electrically nonreactive (Bryce, 1964; Bankes, 1970; George and Anderson, 1986; Gilliland, 1992; Clark *et al.*, 1973). Such characteristics make them ideal for use in many consumer products and industrial procedures (Bryce, 1964). FCs are components of products including household cleaners, leather treatments, insecticides and fire-extinguishing foams; used as surfactants in the aqueous polymerization of fluorinated monomers; and used in industrial processes such as insulating, cooling, wetting, and corrosion inhibition (Bryce, 1964; Bankes, 1970; George and Anderson, 1986; Gilliland, 1992; Clark *et al.*, 1973). Despite the usefulness of these chemicals, some are known to cause mitochondrial inhibition, cholestasis, peroxisome proliferation and tumor formation in rodents (Gilliland and Mandel, 1996; Langely, 1990; Ikeda *et al.*, 1985; Pastoor *et al.*, 1987; Harrision *et al.*, 1988; Abdellatif *et al.*, 1991).

Permadi *et al.*(1993) suggest chain length of FCs influences the severity of effect, finding the greatest significance exhibited by C_8 compounds followed closely by C_{10} compounds, and increasingly less severe consequences exhibited with shorter chained molecules. The work of Feller and Intrasuksri (1993) agreed with that of Permadi *et al.*(1993), and added

that a carboxylic function was important for the stimulation of peroxisome prioliferation. Similar results were reported by Kennedy *et al.* (1998), who analyzed FCs ranging in length from 4-9 carbons for the effect of structure on toxicity. They found C_8 FCs to produce effects at a 10-fold lower dose than C_6 FCs, and short chained FCs to be the least toxic. Although the effects seen in rodents have not been seen in humans, the potential for cumulative and long-term human toxicity resulting from continuous exposure to low concentrations of FCs is of concern (Gilliland and Mandel, 1996; Gilliland, 1992).

PEROXISOMES & PEROXISOME PROLIFERATION

According to Small (1993), peroxisomes (also called microbodies or, in plants, glyoxysomes) are single membrane-limited cytoplasmic organelles present in most eukaryotic cells. The major function of peroxisomes is the β-oxidation of FAs and FA derivatives (Mannaerts and Van Veldhoven, 1993). Peroxisomes contain no DNA; rather, their proteins are synthesized on free polyribosomes in the cell cytosol and imported into pre-existing peroxisomes post-translationally. It is believed that new peroxisomes form by fission from existing peroxisomes.

Peroxisomes have been shown to proliferate following exposure to a diverse class of chemicals referred to as peroxisome proliferators (Green, Issemann and Tugwood, 1993). The mechanism by which this occurs is unclear. Peroxisome proliferation is thought to be mediated by peroxisome proliferator activated receptors (PPARs), nuclear hormone receptors which, upon binding ligand, recognize specific DNA sequence motifs located upstream of the peroxisome proliferator target genes (peroxisome proliferator response

elements (PPREs)), and activate specific gene transcription (Isseman and Green, 1990; Dryer *et al.*, 1993). Due to the diversity of peroxisome proliferators shown to activate PPAR (Green *et al.*, 1993), speculation exists over a direct modulation of PPAR by peroxisome proliferators, and an indirect mechanism is suggested. In addition to chemical and xenobiotic peroxisome proliferators, natural factors such as a high fat diet, starvation and diabetes (Flatmark *et al.*, 1988; Ishii *et al.*, 1980; Ishii, Horie and Suga, 1980; Horie, Fukumori and Suga, 1991; Gottlicher, Widmark and Gustafsson, 1992) have been shown to cause peroxisome proliferation. This correlation between peroxisome proliferation and FA metabolism suggests that PPAR serves an important role in lipid homeostasis (Vanden Heuval, 1996). It is probable, thus, that PPAR activation represents a physiological response to a biological stimulus, likely a factor involved in FA metabolism (Green *et al.*, 1993). Possible stimuli/PPAR ligands include steroids, FAs and derivatives of FA metabolism, and cholesterol metabolites (Green *et al.*, 1993). Target genes include those for acyl-CoA oxidase (Tugwood *et al*, 1992; Feller and Intrasuksri, 1993), L-FABP (Isseman *et al.*, 1992) and P450 $_{IVA1}$ genes (Green *et al.*, 1993).

Significant interest surrounds the issue of peroxisome proliferation because some peroxisome proliferators have been shown to cause hepatocellular carcinomas in laboratory rodents (Moody *et al*, 1991; Vanden Heuval, 1996). The mechanism by which peroxisome proliferators cause cancer in rodents is unknown. They are classified as a novel class of epigenic chemical carcinogen (Vanden Heuvel, 1996), are nonmutagenic in the Ames assay and do not appear to bind DNA (Conway *et* al., 1989; Cohen and Grasso, 1981; Reddy and Lalwani, 1983, Stott, 1988; Reddy and Rao, 1989; Lake *et al.*, 1990).

Multiple mechanisms have been proposed to explain peroxisome proliferator-induced liver tumor formation, including oxidative stress (Reddy and Rao, 1989), enhanced cell replication (Marsman *et al.*, 1988) and promotion of spontaneously formed lesions (Schulte-Hermann *et al.*, 1989). Green *et al.* (1993) propose peroxisome proliferators are "complete carcinogens" which exhibit a combination of initiation (oxidative radical production) and promotion (liver mitogenesis), possibly leading to sustained DNA replication depending on the compound and dose. Doubt about a causal relationship between peroxisome proliferation and carcinogenesis in rodents exists, however, and the relevance to human health is unclear (Tucker and Orton, 1993).

Mammalian species differ in their response to peroxisome proliferators (Lake and Gray, 1985; Rodricks and Turnbull, 1987). Rats are considered strong responders, and guinea pigs and nonhuman primates low to non-responders (Svoboda *et al.*, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986). Slight to no increase in peroxisomes were found in human patients treated with colfibrate (Hanefeld, Kemmer and Kadner, 1983) and fenofibrate (Blümcke *et al.*, 1983), drugs used in the treatment of hypercholesterolemia and known peroxisome proliferators in rodents. Many hypothesize that if a causal relationship does exist between peroxisome proliferation and hepatocarcinogenesis, it is specific to rodents and not a risk to man (Tucker and Orton, 1993).

Other well documented effects of peroxisome proliferators in rodents include inhibition of mitochondria β-oxidation (Elcombe and Mitchell, 1986; Eacho and Foxworthy, 1988;

Foxworthy and Eacho, 1988; Lock, Mitchell and Elcombe, 1989; Wallace, 1998), induction of peroxisomal β-oxidation and ω-oxidation in the ER (Reddy and Lalwani, 1983; Hawkins *et al.*, 1987), induction of L-FABP expression (Bass, Manning and Ockner, 1985; Das, Gourisankar and Mukherjea, 1989; Fleischner *et al.*, 1975), cholestasis (Elcombe and Mitchell, 1986; Foxworthy and Eacho, 1988; Lock *et al.*, 1989; Van Rafelghem *et al.*, 1988) and hepatomegaly (Moody *et al.*, 1991).

FATTY ACID CATABOLISM IN THE HEPATOCYTE

Free fatty acids (FFAs), formed by the breakdown of triacylglycerols stored in adipocytes, are carried in the blood by serum albumin and transported into hepatocytes by what is thought to be a plasma membrane bound fatty acid-binding protein (FABPpm) (Stremmel, Strohmeyer and Berk, 1986; Stremmel *et al.*, 1985). Once in the cell, FFAs are picked up by L-FABP and, under routine conditions, the majority are transported to the mitochondria for β-oxidation, a process by which FAs are degraded to acetyl-CoA by the sequential removal of two carbon segments (Moran and Scrimgeour, 1994). Mitochondrial β-oxidation is coupled to the generation of high energy phosphate bonds via oxidative phosphorylation, and results in the synthesis of ATP and ketone bodies. In order to gain entry into the mitochondria, FA must first be converted to acyl-CoA esters by acyl-CoA synthetases, FA specific enzymes located in the mitochondrial outer membrane (Singh, Derwas and Poulos, 1987). The rate of FA entry into the mitochondria is regulated by carnitine acyl-transferase I, a second enzyme located in the outer membrane of the mitochondria, which converts acyl-CoA esters to acylcarnitines (Murthy and Pande, 1987). Once inside the mitochondria, acylcarnitines are converted back to by acyl-CoA

esters by carnitine acyltransferase II, and degraded by mitochondrial β-oxidation to acetyl-CoA (McGarry and Foster, 1980; Bieber, 1988). Acetyl-CoA is shuttled into the cytosol by the citrate transport system for cholesterol and lipid synthesis (Stryer, 1994). The key factor regulating the rate of mitochondrial β-oxidation is the amount of FA entering the mitochondria which, as stated above, is controlled by carnitine acyl-transferase I. The activity of carnitine acyl-transferase I is controlled by the abundance of malonyl-CoA, the first committed intermediate in FA synthesis (McGarry and Foster, 1980). According to Bass *et al.* (1993), under conditions of increased FA biosynthesis, malonyl-CoA production is increased. Malonyl-CoA is produced from acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase, an enzyme controlled by reversible phosphorylation responding to hormone signals and the presence of fatty acyl-CoA (Moran and Scrimgeour, 1994). When fatty acyl-CoA levels are low, acetyl-CoA carboxylase activity is high. This enhances the conversion of acetyl-CoA to malonyl-CoA. When malonyl-CoA is plentiful, the activity of carnitine-acyltransferase I is limited. This causes the rate of mitochondrial β-oxidation to decrease; FFAs to accumulate; acyl-CoA production and hence cholesterol synthesis to slow; and the rates of alternate routes of FA catabolism, peroxisomal β-oxidation and ω-oxidation in the endoplasmic reticulum (ER), to increase (Lock *et al.*, 1989).

Peroxisomal β-oxidation is normally responsible for catabolizing most, if not all, of the very long chain fatty acids brought into the hepatocyte (Singh *et al.*, 1981; Singh *et al*, 1984; Lazo *et al.*, 1990; Jakobs and Wanders, 1991). This system is also capable of oxidizing medium and long chain FAs and previously activated CoA esters of medium and

long chain dicarboxylic acids. Under normal conditions, however, mitochondrial β-oxidation is the dominant route of catabolism for such substrates (Singh *et al.*, 1987). Peroxisomal β-oxidation proceeds through similar steps as does mitochondrial β-oxidation, however, important differences exist. First, the enzymes used in each process are different proteins (Hashimoto, 1987). Secondly, peroxisomal β-oxidation does not degrade FAs to their two carbon fragments as does mitochondrial β-oxidation; rather, peroxisomal β-oxidation stops after a few cycles, only shortening the carbon chain (Lazarow, 1978; Thomas *et al.*, 1980). Thirdly, peroxisomal β-oxidation is not coupled to an electron transport chain and oxidative phosphorylation as is mitochondrial β-oxidation (Lazarow and de Duve, 1976; Mannaerts *et al.*, 1979). Thus, while mitochondrial β-oxidation produces ATP and ketone bodies, peroxisomal β-oxidation produces hydrogen peroxide and heat. The rate of peroxisomal β-oxidation is thought to be controlled by substrate supply, specifically the activity of acyl-CoA oxidase, which reduces molecular oxygen to hydrogen peroxide in the first step of peroxisomal β-oxidation (Mannaerts *et al.*,1979; Miyazawa *et al.*, 1983). Like substrates for mitochondrial β-oxidation, substrates for peroxisomal β-oxidation must be esterified to their acyl-CoA derivatives; however, peroxisomal β-oxidation is not dependent on carnitine acyl transferase I, as is mitochondrial β-oxidation (Mannaerts and Van Veldhoven, 1993).

ω-Oxidation in the ER, a P450 $_{IVA1}$ mediated process, is responsible for converting monocarboxylic acids to dicarboxylic acids. Dicarboxylic acids are activated in the ER by

dicarboxylyl-CoA synthetase, an enzyme absent in mitochondria and peroxisomes. CoA esters of dicarboxylic acids are almost entirely dependent on mitochondrial β-oxidation for catabolism (Suzuki *et al.*, 1989). The ER also oxidizes bile acid intermediates and is able to esterify very long chain fatty acids (Singh and Poulos, 1988; Lazo *et al.*, 1990). A prerequisite of esterification is activation of FAs to their CoA derivatives (Mannaerts and Van Veldhoven, 1993). ω-Oxidation in the ER is enhanced in cases of FA overload (eg uncontrolled diabetes) or inhibition of mitochondrial β-oxidation (Mortensen and Gregersen, 1981; Golden and Kean, 1984; Mortensen, 1986; Vianey - Liaud *et al.*, 1987); and like peroxisomal β-oxidation, ω-oxidation is not dependent on carnitine acyl transferase I (Mannaerts and Van Veldhoven, 1993).

HYPOTHESIS

As stated above, exposure to FCs leads to mitochondrial inhibition, cholestasis, peroxisome proliferation, and tumor formation in rodents. Three of these endpoints - mitochondrial inhibition, cholestasis and peroxisome proliferation - are directly linked to FA metabolism. This study was designed to test the hypothesis that an initial step in FC-induced peroxisome proliferation is displacement of FAs from L-FABP by FCs. This hypothesis is supported by the fact that L-FABP has been shown to bind nongenotoxic peroxisome proliferators, including certain FCs, *in vitro* (Vanden Heuvel, 1996; Issemann *et al.*, 1992), with relative strengths of binding that parallel their ability to elicit peroxisome proliferation (Brandes *et al.*, 1990; Kanda *et al.*, 1990; Cannon and Eacho, 1991). According to the theory under question, upon displacement of FAs from L-FABP, the intracellular levels of fatty acyl-CoA would decrease. This would increase the activity

of acetyl-CoA carboxylase and enhance the conversion of acetyl-CoA to malonyl-CoA. An increase in the level of malonyl-CoA would repress the activity of carnitine acyltransferase I, and inhibit mitochondrial β-oxidation. ω-Oxidation in the ER would be enhanced, increasing the production of dicarboxylic acids. PPARs would be activated, by the binding of FAs or metabolic intermediates such as dicarboxylic acids, and specific gene transcription of acyl-CoA oxidase, L-FABP and P450 $_{IVA1}$ would be induced. A positive relationship between the amount of L-FABP and the rate of peroxisomal β-oxidation has been found (Appelkvist and Dallner, 1980), and the level of acyl-CoA oxidase is thought to determine the rate of peroxisomal β-oxidation (Mannaerts *et al.*,1979; Miyazawa *et al.*, 1983). Thus, increased transcription of acyl-CoA oxidase and L-FABP would increase rates of peroxisomal β-oxidation and elicit peroxisome proliferation. Induction of P450$_{IVA1}$ genes would further increase the rate of ω-oxidation in the ER. Cholesterol synthesis would eventually cease in response to mitochondrial inhibition and lack of acetyl-CoA production, and decreased esterification by the ER due to decreased acyl-CoA. This would lead to cholestasis. The mechanisms by which carcinogenesis could be induced or promoted will not be discussed.

MATERIALS AND METHODS

MATERIALS

Wyeth-14,643 (WY) was obtained from ChemSyn Science Laboratories, Lexena, KS; FCs were provided by 3M Speciality Chemicals Division, St. Paul, MN; Optifluor LSC-cocktail was obtained from the Packard Instrument Company, Meriden, CT; AMICON YM-5 membrane was purchased from Amicon Corporation, Lexington, MA; BCA Protein Assay was obtained from Pierce Chemical Company, Rockford, IL; and 11-(5-Dimethylaminonapthalenesulphonyl)-undecanoic acid (DAUDA) was purchased from Molecular Probes, Eugene, OR. All other chemicals were obtained from VWR Scientific, West Chester, PA.

ANIMALS AND TREATMENT

Male rats and guinea pigs, 6-8 weeks of age, weighing between 150 and 250 grams were purchased from Charles River Labs, Wilmington, MA . Following an adaptation period of one week after arrival at 3M, animals were weighed, ear-tagged and exposed. The treatment groups consisted of the following:

1. Guinea Pig Vehicle Control - Tween 80, 2% (n = 4);
2. Rat Vehicle Control - Tween 80, 2% (n = 4);
3. Guinea Pig FC1 - FC1 in Tween 80, 2% (n = 4); and,
4. Rat FC1 - FC1 in Tween 80, 2% (n = 4).

All treatments were administered by intraperitoneal (ip) injection. The vehicle control groups were dosed at 5ml / kg body weight 2% Tween 80. The FC1 groups were dosed at

5 ml / kg body weight with a suspension of 32 mM FC1 in Tween-80, 2% (86 mg FC1 / kg body weight) . All animals were housed individually in controlled environments and observed for mortality and clinical signs of toxicity during the first four hours after dosing, at 24 hours, and daily thereafter for the duration of the study. Animals were sacrificed with CO_2 12 days after dosing. Body weights and selected organ weights (liver, kidneys, testes) were recorded at necropsy. Organ tissues and body fluids were stored frozen at -70°C for biochemical analysis or in 10% buffered formaldehyde for subsequent histological analysis. Selected livers were perfused with and stored in gluteraldehyde for future histological analysis by light microscopy.

PURIFICATION OF L-FABP

Protein purification was performed at the University of San Francisco, CA (UCSF) Liver Research Center. Three frozen livers (rat FC1, guinea pig vehicle control, and guinea pig FC1) were shipped in dry ice from 3M to UCSF. These livers and one fresh liver, from a non-treated rat (control) sacrificed at UCSF, were purified.

Frozen livers (approximately 10g each) were thawed and weighed. The fresh liver (approximately 10g) was isolated and perfused with isotonic saline. Each liver was homogenized 30% (w/v) in ice-cold 10mM potassium phosphate buffer, pH 7.4, using a Teflon-glass Potter-Elvehjem tissue homogenizer. The homogenates were centrifuged for 20 minutes at 10,000g in a Sorvall superspeed RC2-B centrifuge maintained at 4°C. The supernatants were removed and subsequently centrifuged for one hour at 38-40,000 rpm (4°C) in a Beckman L7 Ultracentrifuge. The resulting supernatant (cytosol) was labeled with 0.5μCi of 1-^{14}C oleate to trace the L-FABP during purification.

Purification steps were performed in a cold room at 4°C. The cytosol was loaded on a Sephadex G50 M column (5 x 60 cm) equilibrated with 10mM potassium phosphate buffer, pH 7.4. Protein was eluted from the column at a flow rate of approximately 1.4 ml/minute. One hundred fractions were collected (approximately 14.5 ml/fraction). Optifluor LSC-cocktail (5 ml) was added to a 20µl aliquot of each fraction and a Packard Tri-carb 4530 scintillation counter was used to assess L-FABP activity. Fractions with L-FABP activity were pooled and concentrated to approximately 5ml using a vacuum filter apparatus fitted with an AMICON YM-5 membrane. Guinea pig samples (control and FC1 treated) were concentrated and frozen at this point. Rat samples (control and FC1 treated) were further purified as follows.

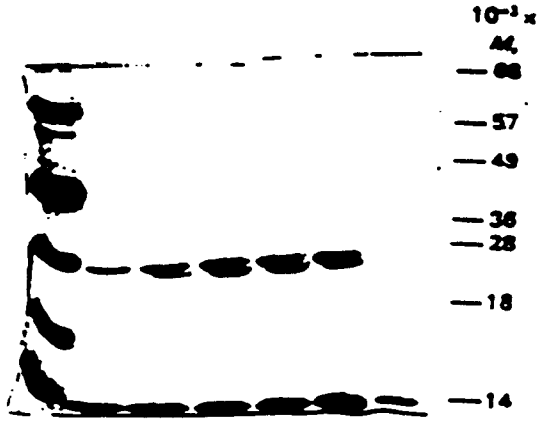
The concentrated solution was loaded on a Sephadex G50 (fine) gel filtration column (2.5 x 45 cm) equilibrated with 10mM potassium phosphate buffer, pH 7.4. The flow rate was approximately 0.9 ml/minute. Sixty fractions were collected at a volume of 3.48ml/fraction. The fractions containing L-FABP activity were pooled and concentrated to approximately 5ml. The concentrated cytosol was dialyzed overnight at 4°C against 30mM Tris-HCl, pH 9, using a Spectrapore Membrane MWCO 3,500. The sample was then applied to a DEAE-cellulose column (Whatman DE-52, 1.25 x 15cm or 2.5 x 15cm) previously equilibrated with 30mM Tris-HCl, pH 9, (degassed). The column was eluted with 30mM Tris-HCl, pH 9, (degassed) followed by a linear gradient of NaCl (0-0.2M) in 30mM Tris-HCl, pH 9. The flow rate was approximatly 1ml/minute. Twenty fractions, 7.8 ml each, were collected.

Homogeneity of the final rat control and rat FC1 fractions was assessed using sodium dodecyl sulfate / polyacrylamide-gel electrophoresis (SDS PAGE) analysis, and confirmed by the appearance of a dominant protein band at molecular weight (MW) 14,000 Daltons (Da.) (Figure 4). Protein concentration of all samples was determined using the bicinchonic acid (BCA) protein assay by Pierce with BSA as the standard (Table 1).

None of the samples were delipidated.

FIGURE 3 - SDS PAGE ANALYSIS OF FRACTIONS FOLLOWING PURIFICATION OF L-FABP FROM RAT LIVER CYTOSOL.
Marker M_r values are shown.

RAT CONTROL L-FABP



RAT FC1 L-FABP

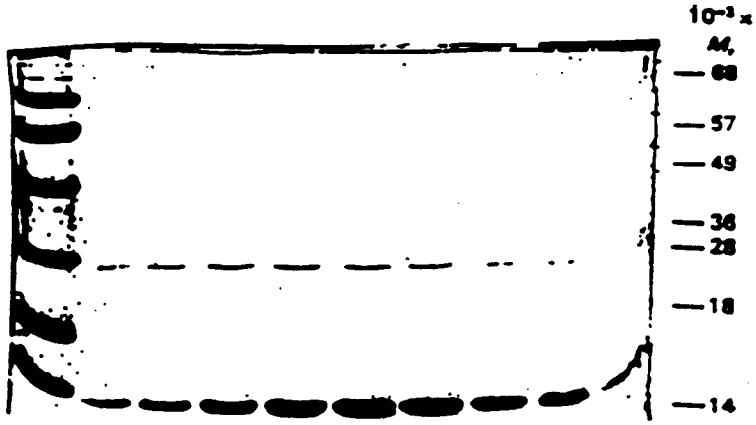


TABLE 1 - PROTEIN CONCENTRATION.
Protein concentration was determined using the BCA protein assay with BSA as the standard. Values are a mean ± standard deviation of 2 trials.

FRACTIONS	TOTAL PROTEIN CONCENTRATION (μg/ml)
PURIFIED	
Rat Control	123.1 ± 13.7
Rat FC1	308.8 ± 1.4
PARTIALLY PURIFIED	
Guinea Pig Vehicle Control	1755.7 ± 10.1
Guinea Pig FC1	1021.0 ± 129.7

FLUORESCENCE MEASUREMENTS

Fluorescence measurements were based on the work of Wilkinson and Wilton (1986). All assays were carried out at room temperature using a slit with of 5nm in a SPEX 1681 0.22m spectrometer, SPEX Industries, Incorporated. A stock solution of DAUDA, 0.1mM, was prepared by slowly adding 50mM potassium phosphate (KH_2PO_4) buffer, pH 7.2, to 1mM DAUDA in methanol. All further dilutions of DAUDA were in 50mM KH_2PO_4, pH 7.4. All dilutions of L-FABP samples were in 50mM KH_2PO_4, pH 7.4. All FCs, WY and oleic acid were dissolved in methanol. All measurements were made after binding had reached equilibrium.

FLUORESCENCE CHARACTERIZATION

Emission and Excitation Maxima and Average Maximum Fluorescence Intensity

The maximum emission and excitation wavelengths (nm) and average maximum fluorescence intensity (FI) (cpm) were determined for 1μM DAUDA binding to each L-

FABP sample. L-FABP, from original undiluted stock, was added to 2ml 1μM DAUDA in aliquots of 1.6 -114μl (depending on the concentration of protein) until no further change in emission or excitation wavelength or FI was detected. The range of protein concentrations analyzed for each L-FABP sample was 0.1μM-3μM. Excitation scans, from 250-400nm using an emission wavelength of 500nm, and emission scans, from 350-600nm upon excitation at 350nm, were performed with each addition. FI (Em. 500nm, Ex., 350nm) was measured following each addition of L-FABP. Curves of FI versus concentration of L-FABP, representing an average ± standard deviation of 3 trials, were constructed for each L-FABP sample. The three highest FI values for each curve were averaged to determine the average maximum FI for DAUDA binding to each sample.

Specific DAUDA Binding

Total binding of DAUDA (0-8μM) to each L-FABP sample was determined by adding 2-20μl aliquots of 0.1mM DAUDA to 2ml 1μM L-FABP. Increased FI (Em. 500nm, Ex., 350nm) due to the binding of DAUDA to protein was measured. Nonspecific binding was assessed by saturating L-FABP binding sites with oleic acid and performing the same titration. Aliquots, 2-20μl, of 0.1mM DAUDA were added to a 2ml solution of 1μM L-FABP and 100μl oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding.

ANALYSIS OF THE EFFECT OF FCS OF L-FABP

Calculation of DAUDA Binding Constants (K_d, B_{max})

Kd and Bmax values were determined for each protein. Specific binding was transformed to units of bound DAUDA (μM) by dividing the specific FI (cpm) by the maximum FI per

1μM DAUDA (cpm) for each L-FABP sample. Computer assisted nonlinear regression (GraFit Version 3, Erithacus Software Limited) was used to construct curves of specific bound DAUDA versus free DAUDA representing an average of 3-6 trials. The following equation was used, Bound = ([L] x Bmax)/(Kd + [L]).

Calculation of Oleic Acid IC_{50}'s

The concentration of oleic acid which inhibits 50% of specific DAUDA binding, the IC_{50}, was calculated for each combination of oleic acid and L-FABP sample. Cuvettes contained 2ml 1μM L-FABP and 1μM DAUDA. Oleic acid, 1mM in 10% methanol, was added in 0.4-20 μl aliquots. FI (cpm) (Em. 500nm, Ex. 350nm) due to the binding of DAUDA to protein following each addition was measured. Curves of percent inhibition of specific DAUDA binding versus oleic acid concentration, representing an average ± standard deviation of 3-6 trials corrected for the effect of methanol, were constructed.

ANALYSIS OF THE POTENCY OF VARIOUS FCS FOR BINDING TO L-FABP

Competitive Binding Experiments - Calculation of IC_{50}'s

Cuvettes contained 2 ml 1μM rat control L-FABP and 1μM DAUDA. Ligands, 1mM (FCs and WY in 100% methanol, and methanol in 50mM KH_2PO_4, pH 7.2), were added in 0.4-20 μl aliquots. FI (cpm) (Em. 500nm, Ex. 350nm) due the binding of DAUDA to protein following each addition was measured. Curves of percent inhibition of specific DAUDA binding versus competitor concentration, representing an average ± standard deviation of 3-6 trials corrected for the effect of methanol were constructed. The concentration of each competitor which inhibited 50% of specific DAUDA binding, the IC_{50}, was calculated.

RESULTS & DISCUSSION

FLUORESCENCE MEASUREMENTS

FLUORESCENCE CHARACTERIZATION

Emission and Excitation Maximum

The emission and excitation spectra of each protein binding DAUDA was analyzed to determine the optimal conditions for the study. The maximum emission and excitation wavelengths for all proteins were approximately 500 and 350nm respectively (Table 2). Values for guinea pig L-FABP samples were slightly higher than those for rat samples. This difference is likely because guinea pig samples were only partially purified; thus, more cellular debris remained to potentially interfere with DAUDA binding, and less L-FABP as a proportion of total protein was present. A "blue shift" in both emission and excitation wavelength occurred upon the addition of each L-FABP sample to DAUDA. Excitation wavelength shifted from approximately 330 to 340nm (Figure 4), and emission wavelength shifted from approximately 550 to 500nm (Figure 5). This shifting of wavelength is characteristic of DAUDA binding to a nonpolar site on L-FABP (Wilkinson and Wilton, 1986). According to Thumser *et al.* (1994a), DAUDA only binds one of the two oleate-binding sites of L-FABP. Due to the stringent conformational requirements of primary site binding (internalized carboxylate/polar group and a U-shaped hydrocarbon/hydrophobic chain), DAUDA most likely binds the secondary site. Similar shifting of fluorescence wavelength and emission and excitation maxima were found by Wilkinson and Wilton (1986) and Thumser, Vosey and Wilton (1996).

TABLE 2 - EMISSION AND EXCITATION MAXIMA.

Fluorescence emission maxima (nm) were measured upon excitation at 350nm. Excitation maxima (nm) were measured using an emission wavelength of 500nm. L-FABP was added to 1μM DAUDA until no further change in emission or excitation wavelength was detected. Each value is an average ± standard deviation of 3 trials.

SAMPLE	EMISSION MAXIMUM (nm)	EXCITATION MAXIMUM (nm)
DAUDA only	551.33 ± 1.53	331.00 ± 1.00
Rat Control L-FABP	502.67 ± 4.62	344.33 ± 2.08
Rat FC1 L-FABP	502.33 ± 4.04	339.67 ± 6.66
Guinea Pig Vehicle Control L-FABP	511.00 ± 7.81	340.67 ± 5.13
Guinea Pig FC1 L-FABP	511.00 ± 7.81	333.67 ± 1.15

FIGURE 4 - EXCITATION MAXIMA.

Fluorescence excitation maxima (nm) were measured using an emission wavelength of 500nm. L-FABP was added to 2ml 1μM DAUDA until no further change in excitation wavelength was detected. Each curve is representative of 3 trials (1μM DAUDA, 3μM L-FABP). KEY : a = Guinea Pig FC1 L-FABP; b = Guinea Pig Vehicle Control L-FABP; c = Rat FC1 L-FABP; d = Rat Control L-FABP.

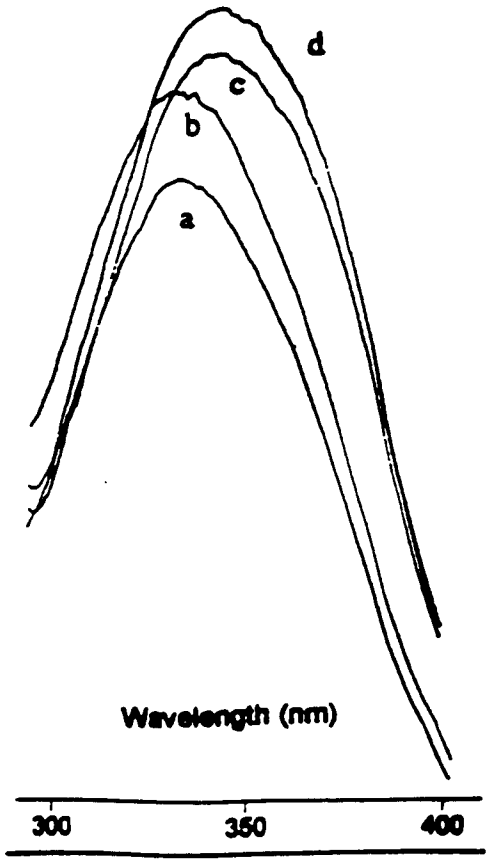
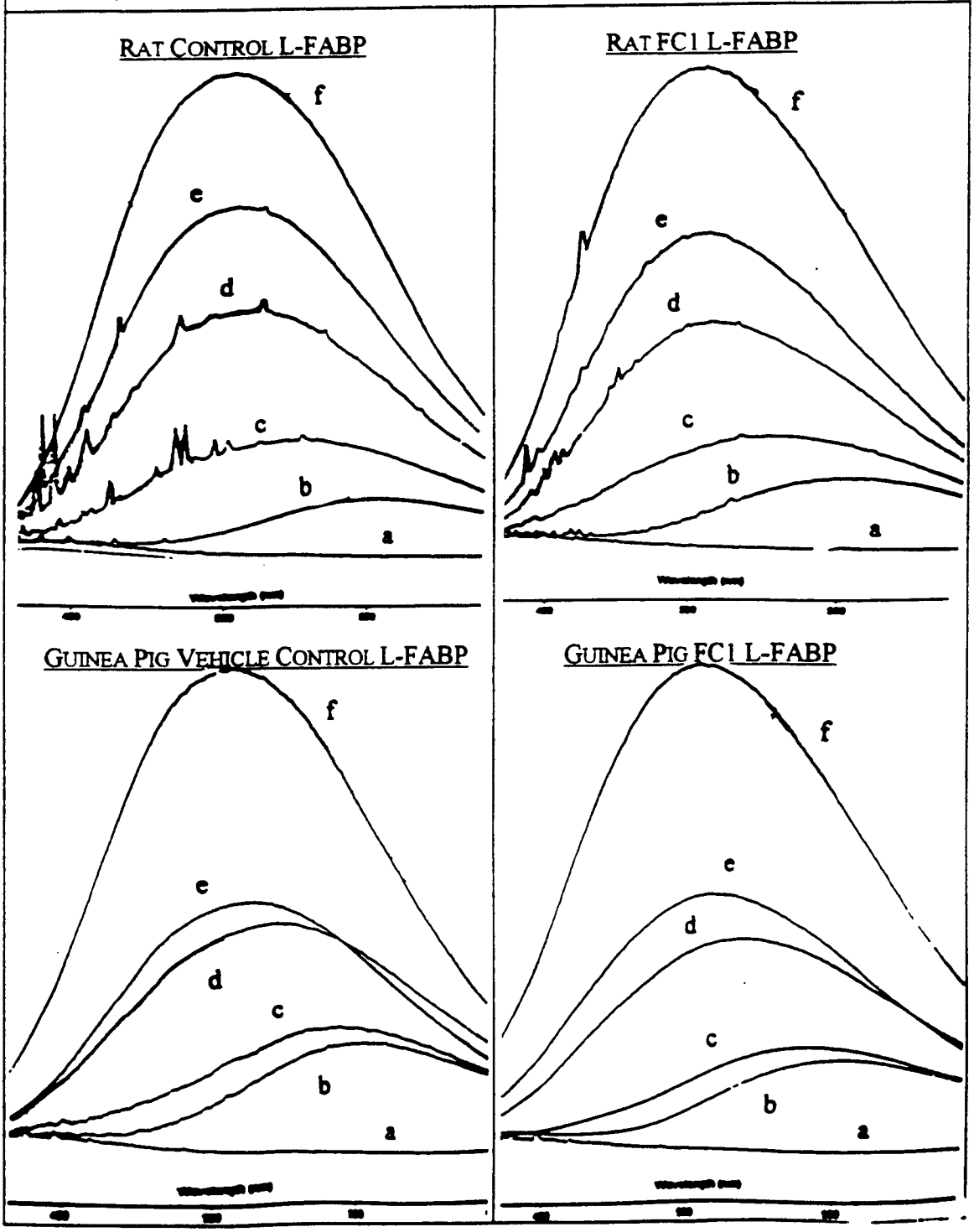


FIGURE 5 - EMISSION MAXIMA.

Fluorescence emission maxima (nm) were measured upon excitation at 350nm. L-FABP was added to 2ml 1μM DAUDA until no further change in emission wavelength was detected. Each curve is representative of 3 trials.

KEY : a = 0 DAUDA, 0 L-FABP, 50mM KH_2PO_4; b = 1μM DAUDA, 0 L-FABP; c = 1μM DAUDA, 0.1μM L-FABP; d = 1μM DAUDA, 0.5μM L-FABP; e = 1μM DAUDA, 1μM L-FABP; f = 1μM DAUDA, 3μM L-FABP.



Average Maximum Fluorescence Intensity

The average maximum FI (cpm) of 1μm DAUDA binding to L-FABP from FC1 treated and non-treated animals did not substantially differ. The average maximum FI was approximately 850,000 cpm for rat L-FABP samples, and 660,000 cpm for guinea pig L-FABP samples (Table 3). Maximum FI was reached following the addition of 1μM rat control L-FABP, 2.5μM rat FC1 L-FABP, 2.5μM guinea pig vehicle control L-FABP and 2μM guinea pig FC1 L-FABP (Figure 6). The lower the affinity of receptor for probe, the higher the concentration of receptor needed for binding, and vice versa (Matthews, 1993). Thus, these data suggest that the rat FC1 L-FABP sample had a decreased affinity for DAUDA as compared to the rat control L-FABP sample, and that the guinea pig FC1 sample had an increased affinity for DAUDA as compared to the guinea pig vehicle control L-FABP sample. The average maximum FI for guinea pig samples was 77.6% lower than that for rat samples. This may be due to the impurity of the guinea pig samples resulting in a high degree of interference in DAUDA binding and a lower concentration of L-FABP as a proportion of total protein.

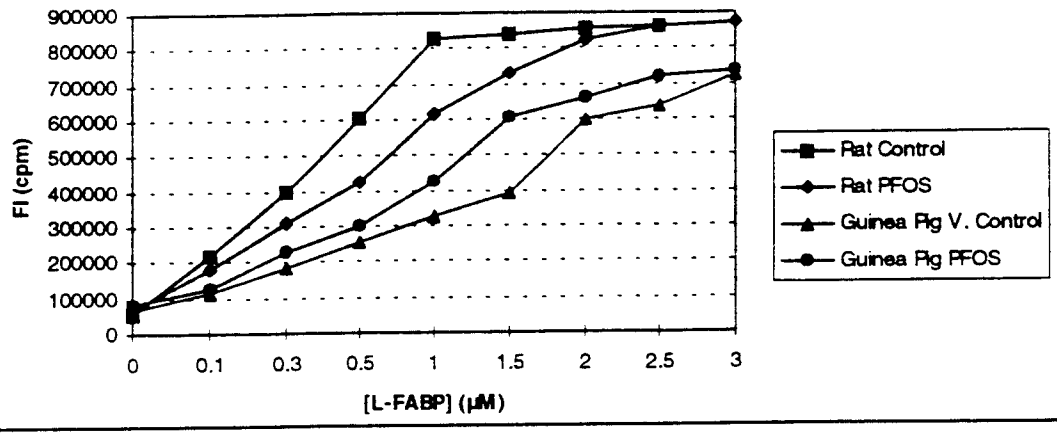
TABLE 3 - MAXIMUM FI OF 1μM DAUDA.

Cuvettes containing 2 ml 1μM DAUDA were titrated with rat and guinea pig L-FABP samples (0.1mM) to determine the maximum FI of 1μm DAUDA binding to each sample. Three trials per protein were performed. Values are the average ± standard deviation of the 3 highest average FI values per protein.

L-FABP SAMPLE	MAXIMUM FI (cpm)
Rat Control	848,093 ±13,639
Rat FC1	852,438 ± 24,776
Guinea Pig Vehicle Control	655,348 ± 63,812
Guinea Pig FC1	669,632 ± 33,619

FIGURE 6 - AVERAGE MAXIMUM FI OF 1μM DAUDA, FI VS [L-FABP].

Cuvettes containing 2 ml 1μM DAUDA were titrated with rat and guinea pig L-FABP samples (0.1mM) to determine the maximum FI of 1μm DAUDA binding to each sample. FI, due to the binding of DAUDA to protein, was measured after each addition. Each curve is an average of 3 trials.



Specific DAUDA Binding

Because of its well documented high affinity for L-FABP (Thumser *et al.*,1994 a,b; Thumser and Wilton, 1994; Thumser *et al.*, 1996; Thumser and Wilton, 1995), oleic acid was chosen as the displacing ligand to determine nonspecific binding of DAUDA to L-FABP. Excess oleic acid was added to occupy all L-FABP binding sites and prevent the specific binding of DAUDA to L-FABP. DAUDA was titrated into the assay and FI, due to nonspecific binding, was observed. Total binding was determined by performing the assay in the absence of oleic acid; specific binding was calculated by subtracting nonspecific binding from total binding. Absolute FI values are given in Table 4. The breakdown of total binding into percent specific and nonspecific binding is shown in Figure 7.

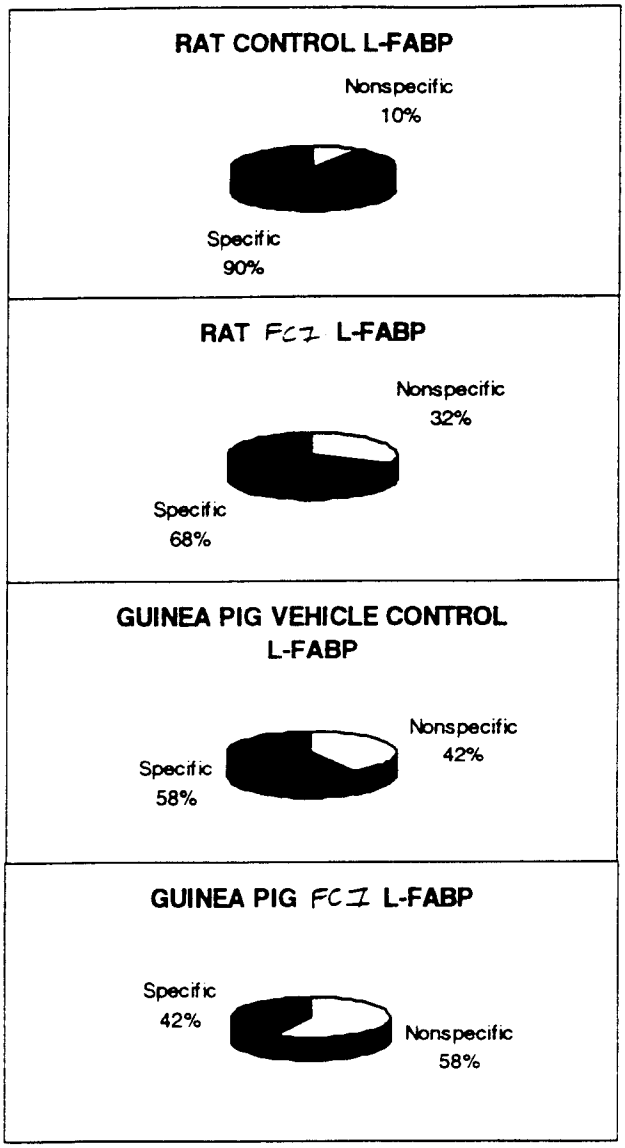
TABLE 4 - 1μM DAUDA BINDING TO 1μM L-FABP.

Total binding of DAUDA to each L-FABP sample was determined by adding aliquots of 0.1mM DAUDA to 2ml 1μM L-FABP. Increased FI due to the binding of DAUDA to protein was measured. Nonspecific binding was measured in the presence of 100μM oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding. Values are an average ± standard deviation of 3-6 trials (cpm).

L-FABP SAMPLE	TOTAL	NONSPECIFIC	SPECIFIC
Rat Control	818,000 ± 124,000	78,000 ± 26,000	740,000 ± 124,000
Rat FC1	302,000 ± 81,000	96,000 ± 21,000	206,000 ± 81,000
Guinea Pig V. Control	219,000 ± 138,000	92,000 ± 23,000	127,000 ± 138,000
Guinea Pig FC1	162,000 ± 15,000	94,000 ± 65,000	68,000 ± 15,000

FIGURE 7 - DAUDA BINDING.

Total binding of DAUDA to each L-FABP sample was determined by adding aliquots of 0.1mM DAUDA to 2ml 1μM L-FABP. Increased FI due to the binding of DAUDA to protein was measured. Nonspecific binding was measured in the presence of 100μM oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding. Values are an average of 3-6 trials.



Specific binding of DAUDA to rat control L-FABP represented approximately 90% of total binding, while specific binding of DAUDA to rat FC1 L-FABP accounted for only 68% of total binding. When a ligand has lower affinity for a receptor, a larger proportion of total binding is nonspecific (Matthews, 1993); thus, these data suggest the rat FC1 L-FABP sample had a three fold lower affinity for DAUDA than the rat control L-FABP sample. This is consistent with the results of the average maximum FI analysis of the rat L-FABP samples, which also suggests the affinity of L-FABP for DAUDA was decreased in the FC1 sample. Less of a difference was seen between the two guinea pig L-FABP samples. Specific binding of 1μM DAUDA to 1μM L-FABP represented 58% of total binding in the guinea pig vehicle control L-FABP sample, and 42% of total binding in the guinea pig FC1 sample. The data suggest the guinea pig FC1 L-FABP sample had lower affinity for DAUDA than the guinea pig vehicle control L-FABP sample. This is not consistent with the results of the average maximum FI analysis, which suggest the relative affinity of the guinea pig FC1 L-FABP sample was higher than that for the guinea pig vehicle control L-FABP sample. This inconsistency may be due to the crudeness of the guinea pig samples giving rise to a high degree of interference in the binding of DAUDA to L-FABP.

The overall crudeness of the guinea pig L-FABP samples, as compared to the rat L-FABP samples, is reflected in a lower absolute total DAUDA binding (cpm), a higher percent nonspecific binding and a lower percent specific binding.

ANALYSIS OF THE EFFECT OF FCS ON L-FABP

Calculation of DAUDA Binding Constants

DAUDA binding constants were calculated for each L-FABP sample. The purpose of this analysis was to assess the effect of FC1 on the functionality of L-FABP; and to confirm the results shown thus far, which suggest the affinity of L-FABP for DAUDA was decreased in FC1 treated rat L-FABP samples, and that guinea pig L-FABP samples were too impure to accurately analyze.

The maximum binding capacity or receptor number (Bmax) of each L-FABP sample and the dissociation constant or affinity (Kd) of each L-FABP sample for DAUDA were determined using computer assisted nonlinear regression (Table 5 and Figure 9). Specific binding of DAUDA to each L-FABP sample was converted to μM by dividing the FI (cpm) due to specific DAUDA binding (Table 4) by the average maximum FI (cpm) per 1μM DAUDA for each L-FABP sample (Table 3).

The Bmax of rat control L-FABP was nearly 1μM. This agrees with the work of Thumser *et al.* (1994 a,b), Thumser and Wilton (1994) and Thumser *et al.* (1996) who all found DAUDA to bind to one of the oleic acid binding sites on L-FABP. The Bmax for the rat FC1 L-FABP sample was approximately 0.35μM, suggesting the capacity of rat FC1 L-FABP to bind DAUDA was nearly one third that of rat control L-FABP. One possible explanation for this decreased capacity is that FC1 is bound to L-FABP in the FC1 sample, rendering fewer available binding sites and allowing less DAUDA to bind. The rigid tail of

FC1 (Zisman, 1964) and strict conformational requirements for primary site binding to L-FABP (Thompson *et al.*, 1997) suggest FC1 binding would occur in the secondary binding site. Another possible explanation is that the vehicle (Tween 80, 2%) somehow affected the binding of DAUDA to L-FABP in the FC1 treated sample. This is unlikely, however, since Tween 80 is a mild, non-ionic detergent, designed to allow solubilized proteins to retain their native structure (Sigma, 1998); and because a low concentration of Tween 80 used (see methods). This does point out, however, the importance of including a vehicle control in the experiment.

The Kds for the rat control L-FABP and rat FC1 L-FABP samples were not notably different and were comparable to the Kd of 0.38 ± 0.02μM found by Thumser *et al.* (1996). Thus, although the data for average maximum FI and percent specific binding suggest a decreased affinity of rat FC1 L-FABP for DAUDA as compared to rat control L-FABP, the Kd and Bmax values suggest the maximum binding capacity/number of binding sites rather than the binding affinity was affected.

The Bmax of the guinea pig vehicle control L-FABP sample was much lower than expected, 0.281μM as compared to approximately 1μM for the rat control L-FABP sample. The Kd for the guinea pig vehicle control L-FABP sample was higher than expected, 0.48μM as compared to approximately 0.3μM for the rat control L-FABP. One possible explanation for these differences in results is that rat L-FABP and guinea pig L-FABP are different. Although the crystal structure of guinea pig L-FABP has not been deduced, sequence identity for FABPs of the same tissue type from different species is

approximately 82-92% (Richieri, Ogata and Kleinfeld, 1994). One can, therefore, expect the binding constants of control guinea pig and rat L-FABP to be comparable. Other possible explanations for this difference are that the vehicle (Tween 80, 2%) had some effect on the L-FABP, or that the crudeness of the guinea pig sample caused a great deal of interference with the binding of DAUDA to L-FABP. As mentioned above, the affect of Tween 80 is presumably minimal; thus, the crudeness of the sample is likely to be to blame for the unexpected binding constants.

As discussed previously, the results for specific binding of DAUDA to guinea pig L-FABP suggest the guinea pig FC1 L-FABP sample had lower affinity for DAUDA than the guinea pig vehicle control L-FABP sample. This suggestion was counter to the results for average maximum FI, which indicate the guinea pig FC1 sample had an increased affinity for DAUDA as compared to the guinea pig vehicle control L-FABP sample. These contradictory data, when combined to calculate a Kd and Bmax for each guinea pig L-FABP sample, resulted in a Kd for the guinea pig FC1 L-FABP sample that was about 4.5 times that of the vehicle control sample, and a capacity of the guinea pig FC1 L-FABP sample that was about twice that of the guinea pig vehicle control L-FABP sample. These results are inconsistent and inconclusive, likely due to the impurity of the guinea pig samples, as discussed above.

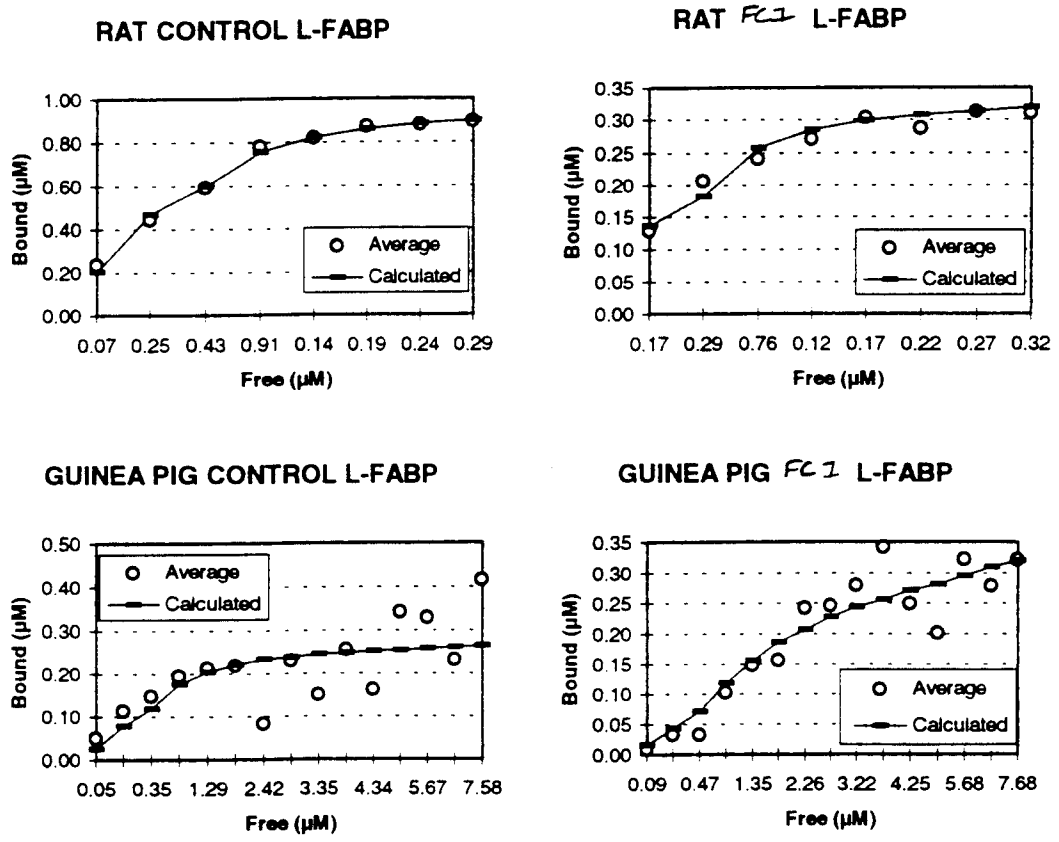
TABLE 5 - DAUDA BINDING CONSTANTS.

Values are an average ± standard deviation of 3 trials as calculated by computer assisted nonlinear regression. Bound = ([L] x Bmax)/(Kd + [L]).

L-FABP SAMPLE	Bmax(μM)	Kd (μM)
Rat Control	0.987 ± 0.016	0.278 ± 0.020
Rat FC1	0.345 ± 0.012	0.260 ± 0.063
Guinea Pig Vehicle Control	0.281 ± 0.043	0.480 ± 0.391
Guinea Pig FC1	0.413 ± 0.060	2.240 ± 0.852

FIGURE 8 - SPECIFIC BOUND VS FREE DAUDA.

Computer assisted nonlinear regression was used to construct curves of specific bound DAUDA versus free DAUDA (Bound = ([L] x Bmax)/(Kd + [L])). Each curve is an average of 3-6 trials. The reduced chi square value for each regression was as follows: rat control L-FABP, 3.83e-16; rat FC1 L-FABP, 7.83e-16; guinea pig vehicle control L-FABP, 6.12e-15; and guinea pig FC1 L-FABP, 1.67e-15.



Calculation of Oleic Acid IC_{50}'s

An IC_{50} was calculated for each combination of oleic acid and L-FABP sample to assess the functionality of L-FABP from FC1-treated and non-treated rats and guinea pigs. Oleic acid, 1mM in 10% methanol, was titrated into 2 ml 1μM L-FABP and 1μM DAUDA. FI (cpm), due the binding of DAUDA to protein, was measured following each addition. Curves of percent inhibition of specific DAUDA binding versus oleic acid concentration were constructed (Figure 9). Table 6 shows the percent inhibition of specific DAUDA binding by 2μM oleic acid and the IC_{50} values for each L-FABP sample. As discussed in the introduction, oleic acid is capable of binding both primary and secondary L-FABP binding sites; and once a ligand has bound the primary site, binding to the secondary site is facilitated (Thompson *et al.*, 1997). This relatively unconstrained binding of oleic acid to L-FABP is reflected in a sharp decrease in FI (cpm) and an increase in DAUDA-specific binding inhibition upon the addition of micro-molar quantities of oleic acid to solutions of L-FABP and DAUDA.

Ninety one percent of specific DAUDA binding to rat control L-FABP was inhibited by 2μM oleic acid; and the IC_{50} of oleic acid for the rat control L-FABP sample was 0.01μM. This percent inhibition was higher than that found by Thumser *et al.* (1994b), Thumser *et al.* (1996) and Thumser and Wilton (1995), of 80.5%, 76% and 80% respectively, under similar conditions. The percent inhibition was much lower (52%) and IC_{50} much higher (0.5μM) for the rat FC1 L-FABP sample as compared to the rat control L-FABP sample. This suggests the ability of L-FABP from the FC1-treated rat to bind oleic acid was decreased. One explanation for this decrease in functionality, is that FC1 was bound to L-

FABP in the FC1 treated sample, rendering fewer sites available to bind oleic acid and requiring more oleic acid to achieve 50% inhibition. This agrees with the results presented for the analysis of average maximum FI, specific DAUDA binding and DAUDA binding constants in rat L-FABP samples.

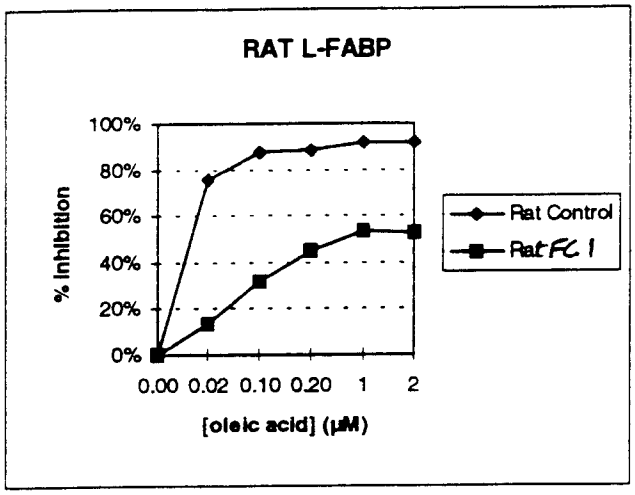
The percent inhibition of specific DAUDA binding to the guinea pig vehicle control L-FABP sample upon addition of 2μM oleic acid was 93%; that for the guinea pig FC1 sample was 63%. This is similar to the results for the rat samples; however, the curves of percent inhibition versus oleic acid concentration for rat and guinea pig samples were very different (Figure 9). The curves for rat samples were hyperbolic while the curves for guinea pig samples were more linear and scattered. The resulting IC_{50} values for both guinea pig L-FABP samples were the same, 0.2μM oleic acid. The data are ambiguous, and are likely due to the crudeness of the guinea pig samples.

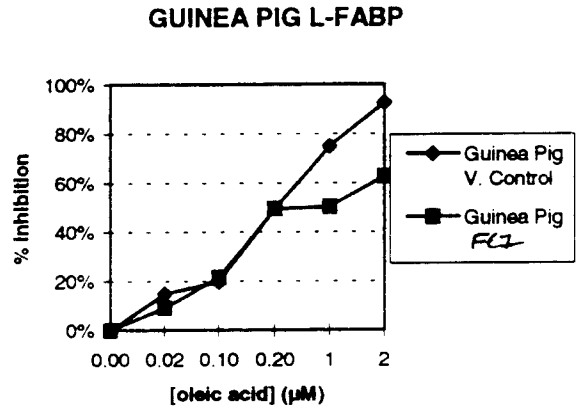
TABLE 6 - OLEIC ACID IC_{50}'S & PERCENT INHIBITION OF SPECIFIC DAUDA BINDING.
The IC_{50} of oleic acid for each L-FABP sample was calculated by adding oleic acid, 1mM in 10% methanol, to a solution of 1μM L-FABP and 1μM DAUDA. Values are IC_{50}'s of oleic acid for each L-FABP sample and percent inhibition of specific DAUDA binding by 2μM oleic acid.

L-FABP Sample	Oleic Acid IC_{50} (μM)	% Inhibition by 2μM oleic acid
Rat Control	0.01	91
Rat FC1	0.5	52
Guinea Pig Vehicle Control	0.2	93
Guinea Pig FC1	0.2	63

FIGURE 9- % DAUDA INHIBITION VS [OLEIC ACID].

Oleic acid, 1mM in 10% methanol, was added to a solution of 1μM L-FABP and 1μM DAUDA. Curves represent an average of 3-6 trials corrected for the effect of methanol.





ANALYSIS OF THE POTENCY OF FCS FOR BINDING TO L-FABP

Competitive Binding Experiments - Calculation of IC_{50}'s

An IC_{50} of each FC, WY and methanol was calculated for DAUDA binding to the rat control L-FABP sample. Each ligand (1mM, in 100% methanol or 50mM KH_2PO_4, pH 7.2) was titrated into 2ml 1μM rat control L-FABP and 1μM DAUDA. The addition of

competitor to L-FABP and DAUDA induced a shift of emission wavelength back towards the curve of DAUDA with no L-FABP (Figure 10). This is indicative of DAUDA being displaced from a nonpolar binding site on L-FABP, such as the secondary binding site. To varying degrees, similar shifting and decreases in FI occurred upon addition of each competitor. FI (cpm), due the binding of DAUDA to protein, was measured following each addition of competitor; and curves of percent inhibition of specific DAUDA binding versus ligand concentration constructed (Figure 11). Table 8 gives the percent inhibition of specific DAUDA binding by 10μM competitor and the IC_{50} values for each competitor.

FC1 was the strongest inhibitor of specific DAUDA binding, with 69% inhibition upon the addition of 10μM and an IC_{50} of 4.9μM. FC4 was the next strongest, with 51% inhibition upon the addition of 10μM and an IC_{50} of 9.7μM. Wyeth followed FC4, with 50% inhibition upon the addition of 10μM, and an IC_{50} of 10μM. FC3 and FC2 both inhibited 43% of specific DAUDA binding upon the addition of 10μM and had IC_{50}'s greater than 10μM.

Thumser and Wilton (1996) suggest that ligands for L-FABP need to have both a hydrophobic and a hydrophilic domain. Each FC and WY fits this description to varying degrees. Due to the rigid tail of the FCs and the bulky structure of WY, it is likely that binding of each of these competitors to L-FABP occurs in the secondary binding site on L-FABP. It is conceivable that the FCs bind with their rigid hydrophobic CF tails, analogous to the flexible CH tail of oleic acid, in the secondary binding site and that the polar head groups of FCs are solvent exposed as is the carboxylate group on oleic acid.

To reason by structure, the varying potencies of each FC to binding L-FABP is difficult. Thompson *et al.* (1997) suggest that anything larger than a C_{14} molecule may require prior binding in the primary site. As was mentioned in the introduction, C_8 and C_{10} FCs are the most potent peroxisome proliferators. It may be the case that C_8 and C_{10} molecules bind most readily to the secondary binding site on L-FABP, not requiring a molecule to be bound in the primary binding site. All of the FCs examined in the present study were C_8 molecules; thus, an analysis of chain length cannot be undertaken at this time. The disparate potencies of binding L-FABP between FCs do not appear to be due to differences in polarity and hydrogen bonding ability. For example, FC2, having a very polar carboxylate head group, and capable of extensive hydrogen bonding, has a higher IC_{50} than FC4, which has a less polar head group and less ability to hydrogen bond (see Figure 1).

The binding of WY to the secondary binding site on L-FABP is difficult to reason. In place of the hydrophobic tail, present on FCs and oleic acid, WY has two aeromatic rings. The conformational flexibility of L-FABP may accommodate this bulky structure internally, and position the carboxylate externally, in a solvent exposed manner able to participate in hydrogen bonding. Thompson *et al.* (1997) also speculate that a third binding site, located completely on the exterior of L-FABP, may exist. Only the first few carbons of a molecule binding to such a site would be bound in an organized fashion, the remaining portion of the ligand would be disordered. This, although highly speculative, may be the site of WY binding to L-FABP.

Methanol was chosen as the negative control because a non-peroxisome proliferator, similar in structure to the FCs being researched, has not been identified. Upon the addition of 10μM methanol, 21% of specific DAUDA binding was inhibited. The IC_{50} of methanol was much greater than 10μM. It is reasonable to suggest methanol is capable of weakly binding the secondary L-FABP binding site, with the CH_3 group internally located and the OH group exposed to the exterior of the protein. The structure of methanol renders it an unlikely candidate for looping into a U-shape and participating in primary site binding.

FIGURE 10 - CHROMATOGRAM.

The addition of competitor to L-FABP and DAUDA induced a shift of emission wavelength back towards the curve of DAUDA with no L-FABP. This is indicative of DAUDA being displaced from a nonpolar binding site on L-FABP. To varying degrees, similar shifting and decreases in FI occurred upon addition of each competitor. The example shown is for FC2 binding rat control L-FABP.

KEY : a = 1μM DAUDA, 1μM L-FABP; b = 1μM DAUDA, 1 L-FABP, 10μM FC2; c = 1μM DAUDA, 1μM L-FABP, 15μM FC2; d = 1μM DAUDA, 1μM L-FABP, 20μM FC2; e = 1μM DAUDA; f = 0 DAUDA, 0 L-FABP, 50mM KH_2PO_4.

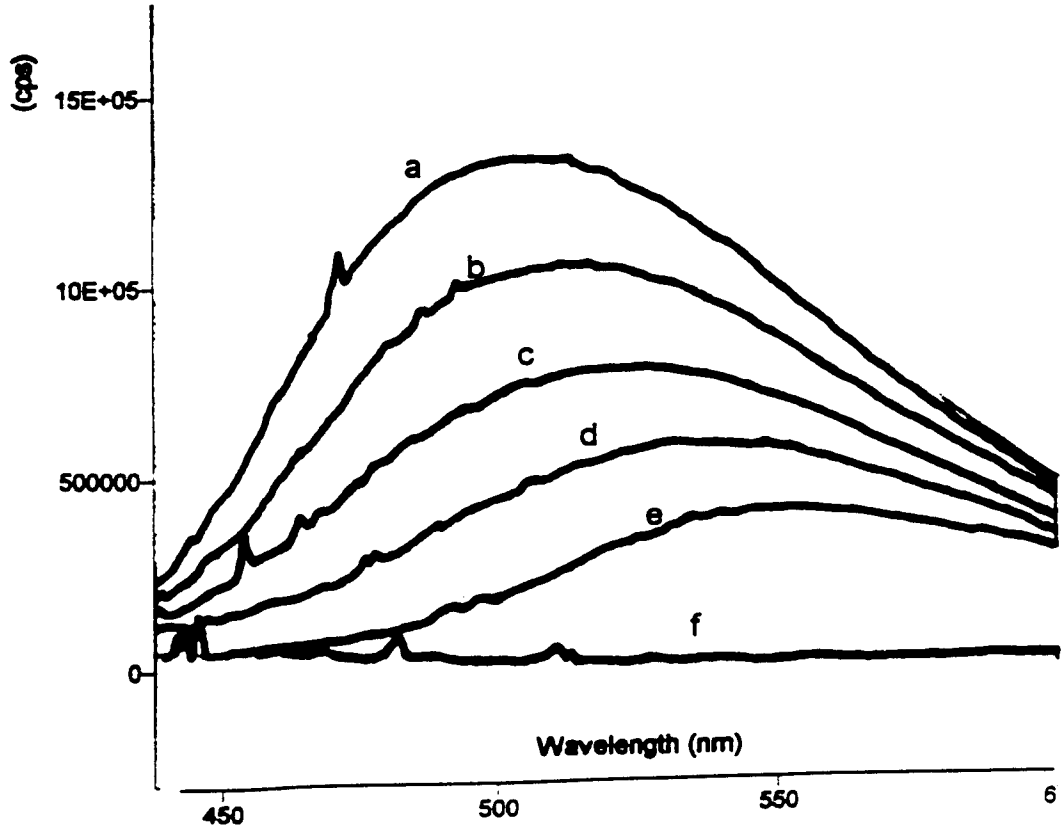


FIGURE 11 - % INHIBITION OF SPECIFIC DAUDA BINDING VS [COMPETITOR].
Each competitor, 1mM, was added to 1μM L-FABP and 1μM DAUDA. Measurements of FI were made upon each addition. Curves represent an average of 3-6 trials corrected for the effect of methanol.

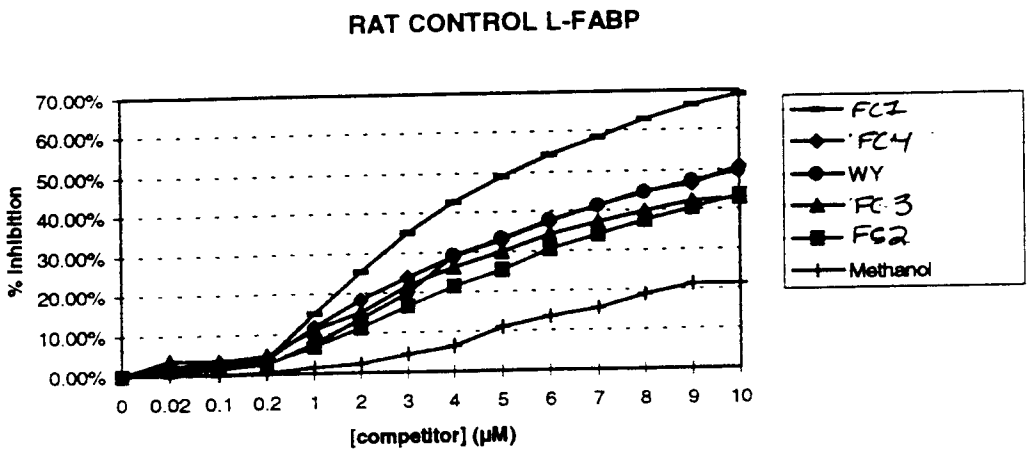


TABLE 8 - IC_{50}'S OF FCS AND WY & PERCENT INHIBITION OF SPECIFIC DAUDA BINDING.
The IC_{50} of each competitor for the rat L-FABP sample was calculated by adding competitor (1mM) to 1μM L-FABP and 1μM DAUDA. Values are the IC_{50}'s of each competitor and % inhibition of specific DAUDA binding by 10μM of each competitor.

COMPETITOR	IC_{50} (μm)	% INHIBITION
FC1	4.9	69
FC4	9.7	51
WY	10	50
FC3	>10	43
FC2	>10	43
METHANOL	>10	21

CONCLUSIONS & FUTURE DIRECTIONS

This study was designed to investigate the hypothesis that certain Fluorocarbons (FCs) bind to liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event in peroxisome proliferation. To examine this hypothesis, the kinetics of FA and FC binding to L-FABP were investigated using DAUDA, a fluorescently labeled octanoic acid analogue. L-FABP from male rats (considered to be strong responders to peroxisome proliferators) and male guinea pigs (considered to be weak or non-responders to peroxisome proliferators) (Svoboda *et al.*, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986) were examined.

The first goal was to assess the effect of FCs on L-FABP function as evaluated by the ability of DAUDA to bind to L-FABP isolated from rats and guinea pigs treated and not treated with FC1 *in vivo*. Results show a decreased maximum binding capacity of L-FABP from FC1-treated rats without an increase in Kd. This was demonstrated by a lower Bmax, higher oleic acid IC_{50} and unchanged Kd in L-FABP from rats treated with FC1 as compared to samples from control rats. These results are likely due to FC1 binding to the secondary binding site on L-FABP; thus preventing the binding of DAUDA to L-FABP. Results for guinea pig L-FABP samples were inconclusive. This is presumably because guinea pig samples were only partially purified, resulting in a high degree of interference from remaining cellular debris and a lower concentration of L-FABP, as a proportion of total protein, as compared to the more highly purified rat samples. A second attempt will be made to analyze the difference in response to FCs seen in rodent and guinea pig L-FABP. This analysis will be performed on fully purified L-FABP samples.

The second goal of the study was to assess the potency of the various FCs for binding to L-FABP, as suggested by IC_{50} values. Results indicate the most potent L-FABP binder is FC1, followed by FC4, WY and (with equal IC_{50}s) FC3 and FC2. Binding of FCs to L-FABP likely occurs in the secondary binding site; and the variance in potency is likely due to structural differences.

Future work will focus on correlating new data with the results of this study, and relating the effects seen in rodents and guinea pigs to the relevance they pose to human health. The possibility of completing a new set of kinetics assays using a probe capable of binding both L-FABP binding sites will be looked into. This would help characterize the interaction of DAUDA with L-FABP and create a new set of results for analysis. Human L-FABP will be examined, and the relevance to human health of FC induced peroxisome proliferation in rodents will be investigated. FCs of different chain length will be investigated, and the effect of chain length on ability to bind L-FABP analyzed. The ability of each FC to induce peroxisome proliferation in rodent L-FABP will be assessed using the method of Lazarow and de Duve (1976). An attempt will be made to correlate this capacity with the relative potency of each FC to bind L-FABP, as determined in the current study. Electro-Spray Mass Spectrometry analysis on the same L-FABP samples as analyzed in this study is currently underway. The objective is to quantify FC1 bound to L-FABP from FC1 treated animals, correlate this with the decrease in L-FABP capacity observed in this study and with the ability of FC1 to induce peroxisome proliferation in rodents. The long range goal is to refine the correlation between the amount of FC bound to L-FABP with peroxisome

proliferation, and develop a biomarker for peroxisome proliferation based on bound FC levels. This biomarker would be used as a method of screening FCs for their ability to induce peroxisome proliferation.

REFERENCES

Abdellatif A., Preat V., Taper H. and Roberfroid M. (1991). The modulation of rat liver carcinogensis by perfluorooctanoic acid, a peroxisome proliferator. *Toxicol. Appl. Pharmacol.*, **111**: 530-7.

Appelkvist E. and Dallner G. (1980). Possible involvement of fatty acid-binding protein in peroxisomal β-oxidation of fatty acids. *Biochim. Biophys. Acta*, **617**: 156-60.

Bankes R. (1970). Fluorocarbons and their Derivatives. MacDonald Technical and Scientific, London.

Bass N. (1985). Function and regulation of hepatic and intestinal fatty acid-binding proteins. *Chem. Phys. Lipids*, **38**: 95-114.

Bass N. (1988). The cellular fatty acid binding proteins: Aspects of structure, regulation and function. *Int. Rev. Cytol.*, **III**: 143-84.

Bass N., Kaikaus R. and Ockner R. (1993). Physiology and molecular biology of hepatic cystosolic fatty acid-binding protein. Chapter 28 in Hepatic Transport and Bile Secretion: Physiology and Pathophysiology. Ed. Tavoloni, N. and Berk, P.D. Raven Press, Ltd., New York.

Bass N., Manning J. and Ockner R. (1985). Turnover and short-term regulation of fatty acid-binding protein in liver. *J. Biol. Chem.*, **260**: 9603-7.

Bieber L. (1988). Carnitine. *Annu. Rev. Biochem.*, **57**: 261-83.

Blümcke S., Schwartzkopff W., Lobeck H., Edmondson N., Prentice D. and Blane G. (1983). Influence of fmofibrate on cellular and subcellular liver structure in hyperlipidemic patients. *Atherosclerosis*, **46**: 105-16.

Brandes R., Kaikaus R., Lysenko N., Ockner R. and Bass N. (1990). Induction of fatty acid binding protein by peroxisome proliferators in primary hepatocyte cultures and its relationship to the induction of peroxisomal beta-oxidation. *Biochim. Biophys. Acta*, **1034**: 53-61.

Bryce H. (1964). Industrial and utilitarian aspects of fluorine chemistry. pp. 297-492 in Fluorine Chemistry. Ed. Simons J., New York: Academic Press.

Cannon J. and Eacho P. (1991). Interaction of LY171883 and other peroxisome proliferators with fatty-acid-binding protein isolated from rat liver. *Biochem. J.*, **280**: 387-91.

Chan L., Wei C-F. Li W., Yang C-Y, Ratner P., Pownall H., Gotto Jr. A., Smith L. (1985). Human liver fatty acid binding protein cDNA and amino acid sequence. Functional and evolutionary implications. *J. Biol. Chem.*, **260**: 2629-32.

Cohen A. and Grasso P. (1981). Review of the hepatic response to hypolipidaemic drugs in rodents and assessment of its toxicological significance to man. *Food Cosmet. Toxicol.*, **19**: 585-605.

Clark L., Becattini F., Kaplan S., Obrock U., Cohen D. and Becker C. (1973). Fluorocarbons having a short dwell time in the liver. *Science*, **181**: 680-2.

Conway J., Tomazzewski K., Olson M., Cattley R., Marsman D. and Popp J. (1989). Relationship of oxidative damage to the hepatocarcinogenicity of the peroxisome proliferators di-(2-ethylhexyl)phthalate and Wy-14, 643. *Carcinogenesis*, **10**: 513-19.

Das T., Gourisankar S. and Mukherjea M. (1989). Human fetal liver fatty acid binding proteins. Role on glucose-6-phosphate dehydrogenase activity. *Biochim. Biophys. Acta*, **1002**: 164-72.

Dryer C., Keller H., Mahfoudi A., Laudet V., Krey G. and Wahli W. (1993). Positive regulation of the peroxisomal β-oxidation pathway by fatty acids through activation of peroxisome proliferator activated receptors (PPAR). *Biochem. Biophys. Res. Commun.*, **77**: 67-76.

Eacho P. and Foxworthy P. (1988). Inhibition of hepatic fatty acid oxidation by benzafibrate and bezafibroyl CoA. *Biochem. Biophys. Res. Commun.*, **157**: 1148-53.

Elcombe C. and Mitchell A. (1986). Peroxisome proliferation due to di(2-ethylhexyl) phthalate (DEHP): Species differences and possible mechanisms. *Environ. Health Perspect.*, **70**: 211-19.

Feller D. and Intrasuksri U. (1993). Structural requirements for peroxisome proliferation by phenoxyacetic and fatty acid analogues in primary cultures of rat hepatocytes. Chapter 16 in Peroxisomes - Biology and Importance in Toxicology and Medicine. Ed. Gibson, G. and Lake, B. Taylor and Francis, Washington, DC.

Flatmark T., Nilsson A., Kvannes J., Eikhom T., Fukami M., Kryvi H. and Christiansen E. (1988). On the mechanism of induction of the enzyme systems for peroxisomal β-oxidation of fatty acids in rat liver by diets rich in partially hydrogenated fish oil. *Biochim. Biophys. Acta*, **962**: 122-30.

Fleischner G., Meijer D., Levine W., Gatmaitan Z., Gluck R. and Aris I. (1975). Effect of hypolipidemic drugs, nafenopin and clofibrate, on the concentration of ligandin and Z protein in rat liver.*Biochem. Biophys. Res. Commun.*, **67**: 1401-7.

Foxworthy P. and Eacho P. (1988). Inhibition of hepatic fatty acid oxidation at carnitine palmitoyltransferase I by the peroxisome proliferator 2-hydroxy-3-propyl-4-[6-(tetrazol-5-yl)hexyloxy]acetophenone. *Biochem. J.*, **252**: 409-14.

George M. and Anderson M. (1986). Toxic effects of perfluoro-*n*-decanoic acid in rats. *Toxicol. Appl. Pharmac.*, **85**: 169-80.

Gilliland F. (1992). Fluorocarbons and human health: Studies in an occupational cohort. PhD. Dissertation, University of Minnesota, Minneapolis, MN.

Gilliland F. and Mandel J. (1996). Serum perfluorooctanoic acid and hepatic enzymes, lipoproteins, and cholesterol: A study of occupationally exposed men. *Am. J. Ind. Med.*, **29**: 560-8.

Golden K. and Kean E. (1984). The biogenesis of dicarboxylic acids in rats given hypoglycin. *Biochim. Biophys. Acta*, **794**: 83-8.

Gottlicher M., Widmark E., Li Q. and Gustafsson J. (1992). Fatty acids activate chimera of the colfibrate acif activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA*, **89**: 4653-7.

Gordon J., Alpers D., Ockner R. and Strass A. (1982). The nucleotide sequence of rat liver fatty acid -binding protein mRNA. *J. Biol. Chem.*, **258**: 3356-63.

Green S., Issemann I. and Tugwood J. (1993). The molecular mechanism of peroxisome proliferator action. Chapter 4 in Peroxisomes: Biology and Importance in Toxicology and Medicine. Ed. Gibson, G. and Lake, B. Taylor and Francis, Washington, DC.

Hanefeld M., Kemmer C. and Kadner E (1983). Relationship between morphological changes and lipid-lowering action of *p*-chlorphenoxyisobuteric acid (CPIB) on hepatic mitochondria and peroxisomes in man. *Atherosclerosis*, **46**: 239-46.

Harrison E., Lane J., Luking S., Van Rafelghem M. and Anderson M. (1988). Perfluoro-n-decanoic acid: Induction of peroxisomal β-oxidation by fatty acid with dioxin-like toxicity. *Lipids*, **23**:115-9.

Hashimoto T. (1987). Comparison of enzymes of lipid β-oxidation in peroxisomes and mitochondria. pp. 97-104 in Peroxisomes in Biology and Medicine. Ed. Fahimi H and Sies H. Springer-Verlag, Berlin.

Hawkins J., Jones W., Bonner F. and Gibson G. (1987). The effects of peroxisome proliferators on microsomal, peroxisomal and mitochondrial enzyme activities in the liver and kidney. *Drug Metab. Rev.*, **18**: 441-515.

Horie S., Fukumori N. and Suga T. (1991). Induction of hepatic peroxisomes by a new non-carboxylic-containing drug, bifonazole. *Toxicol. Lett.*, **55**: 249-54.

Ikeda T., Aiba K., Fukuda K. and Tanaka M. (1985). The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolic inert derivatives of fatty acids. *J. Biochem.*, **98**: 475-82.

Ishii H., Fukumori N., Horie S. and Suga T. (1980). Effects of fat content in the diet on hepatic peroxisomes in the rat. *Biochim. Biophys. Acta*, **617**: 1-11.

Ishii H., Horie S. and Suga T. (1980). Physiological role of peroxisomal β-oxidation in liver of fasted rats. *J. Biochem. (Tokyo)*, **87**: 1855-8.

Issemann I., and Green S. (1990). Activation of a member of the steroid hormone subfamily by peroxisome proliferators. *Nature*, **347**: 645-50.

Issemann I., Prince P., Tugwood J. and Green S. (1992). A role of fatty acids and liver fatty acid-binding protein in peroxisome proliferation? *Biochem. Soc. Trans.*, **20**: 824-7.

Jakobs B. and Wanders R. (1991). Conclusive evidence that very long chain fatty acids are oxidized exclusively in peroxisomes in human skin fibroblasts. *Biochem. Biophys. Res. Commun.*, **178**: 842-7.

Kaikaus R., Bass N. and Ockner R. (1990). Functions of fatty acid-binding proteins. *Experientia* **46**: 617-30.

Kanda T., Iseki S., Hitomi M., Kimura H., Odani S., Kondo H., Matsubara Y., Muto T. and Ono T. (1989). Purification and characterization of a fatty-acid-binding protein from the gastric mucosa of rats. Possible identity with heart fatty-acid-binding protein and its parietal cell localization. *Eup. J. Biochem.*, **185**: 27-33.

Khan S. and Sorof S. (1990). Preferential binding of growth inhibitory prostaglandins by the target protein of a carcinogen. *Proc. Natl. Acad. Sci. USA*, **87**: 9401-5.

Kennedy G., Keller D., Biegel L. and Brock W. (1998). Repeated-dose toxicity of fluorinated acids in rats. *The Toxicologist 37th Annual* Meeting, **37(1-S)**: 371.

Lake B. and Gray T. (1985). Species differences in hepatic peroxisome proliferation. *Biochem. Soc. Trans.*, **13**: 859-62.

Lake B., Gray T., Smith A. and Evans J. (1990). Hepatic peroxisome proliferation and oxidative stress. *Biochem. Soc. Trans.*, **18**: 94-7.

Langely A. (1990). Effects of perfluoro-*n*-decanoic acid on the respiratory activity of isolated rat liver mitochondria. *J. Toxicol. Environ. Health*, **29**: 329-36.

Lazarow P. (1978). Rat liver peroxisomes catalyze the β-oxidation of fatty acids. *J. Biol. Chem.*, **253**: 1522-8.

Lazarow P. and de Duve C. (1976). A fatty acyl-CoA oxidizing system in rat liver peroxisomes: enhancement by colfibrate, a hypolipidemic drug. *Natl. Acad. Sci. USA*, **73**: 2043-6.

Lazo O., Contreras M., Yoshida Y., Singh A., Stanley W., Weise M. and Singh Y. (1990). Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases. *J. Lipid Res.*, **31**: 583-95.

Levi A., Gatmaitan Z. and Area I. (1969). Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromopthalein, and other anions. *J. Clin. Invest.* **48**: 2156-67.

Lock E.. Mitchell A. and Elcombe C. (1989). Biochemical mechanisms of induction of peroxisome proliferation. *Rev. Pharmacol. Toxicol.*, **29**: 145-63.

Mannaerts G., Debeer L., Thomas J. and De Schepper P. (1979). Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and colfibrate treated rats. *J. Biol. Chem.*, **254**: 4585-95.

Mannaerts G. and Van Veldhoven P. (1993). Metabolic role of mammalian peroxisomes. Chapter 2 in Peroxisomes: Biology and Importance in Toxicology and Medicine. Ed. Gibson, G. and Lake, B. Taylor and Francis, Washington, DC.

Marsman D., Cattley R., Conway J. and Popp J. (1988). Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio)acetic (Wy-14,643) in rats. *Cancer Res.*, **48**: 6739-44.

Matthews J. (1993). Fundamentals of Receptor, Enzyme, and Transport Kinetics. CRC Press, Inc., Boca Raton, Florida, USA.

McGarry J. and Foster D. (1980). Regulation of hepatic fatty acid oxidation and ketone body production. *Ann. Rev. Biochem.*, **49**: 395-420.

Miyazawa S., Ozasa H., Osumi T. and Hashimoto T. (1983). Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. *J. Biochem. (Tokoyo)*, **94**: 529-42.

Moody D., Reddy J., Lake B., Popp J. and Reese D. (1991). Peroxisome proliferation and nongenotoxic carcinogens. *Fundam. Appl. Toxicol.*, **16**: 233-48.

Moran L. and Scrimgeour K. (1994). Biochemistry Resource Book. Ed. Pratt C. Neil Patterson Publishers Prentice Hall, Englewoods Cliffs, NJ.

Mortensen P. (1986). C_6-C_{10}-Dicarboxylic acids in liver and kidney tissue in normal, diabetic ketotic and colfibrate-treated rats. *Biochim. Biophys. Acta*, **878**: 14-9.

Mortensen P. and Gregersen N. (1981). The biological origin of ketotic dicarboxylic acidemia, *Biochim. Biophys. Acta*, **666**: 394-404.

Murthy M. and Pande S. (1987). Malonyl-CoA binding site and the overt carnitine palmitoyl-transferase activity reside on the opposite sides of the outer mitochondrial membrane. *Proc. Natl. Acad. Sci. USA*, **84**: 378-82.

Ockner R., Manning J., Poppenhausen R. and Ho. W. (1972). A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science*, **177**: 56-8.

Orton T., Adam H., Bentley M., Holloway B. and Tucker M. (1984). Clobuzarit: Species differences in the morphological and biochemical response of the liver following chronic administration. *Toxicol. Appl. Pharmacol.*, **73**: 138-51.

Pastoor T., Lee K., Perri M. and Gillies P. (1987). Biochemical and morphological studies of ammonium perfluorooctane-induced hepatomegaly and peroxisome proliferation. *Exp. Mol. Pathol.*, **47**: 98-109.

Permadi H., Lundgren B., Anderson K., Sunberg C. and DePierre J. (1993). Effects of perfluoro fatty acids on peroxisome proliferation and mitochondrial size in mouse liver: Dose and time factors and effect of chain length. *Xenobiotica* **23, 7**: 761-70.

Reddy J., Lalwani N. (1983). Carcinogenesis by hepatic peroxisome proliferators: Evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *CRC Crit. Rev. Toxicol.*, **12**: 1-58.

Reddy J. and Rao M. (1989). Oxidative DNA damage caused by persistent peroxisome proliferation: Its role in hepatocarcinogenesis. *Mutat. Res.*, **214**: 63-8.

Richieri G., Ogata R. and Kleinfeld (1994). Equilibrium constants for the binding of fatty acids with fatty acid-binding proteins from adipocyte, intestine, heart, and liver measured with the fluorescent probe ADIFAB. *J. Biol. Chem.*, **269 (39)**: 23918-30.

Rodricks J. and Turnbull D. (1987). Interspecies differences in peroxisomes and peroxisome proliferation. *Toxicol. Ind. Health*, **3**: 197-213.

Rolf B., Oudenampsen-Kruger E., Borchers T., Faergemann N., Knudsen J., Lezius A. and Spener F. (1995). Analysis of the ligand binding properties of recombinant bovine liver-type fatty acid binding protein. *Biochim. Biophys. Acta*, **1259**: 245-53.

Schulte-Hermann R., Kraupp-Grasl B., Bursch W., Gerbracht U. and Timmermann-Trosiener I. (1989). Effects of non-genotoxic hepatocarcinogens phenobarbital and nefenopin on phenotype and growth of different populations of altered foci in rat liver. *Toxicol. Pathol.*, **17**: 642-50.

Sigma (1998). Internet Address: http://www.sial.com/sigma/fields/tween80.htm

Singh H., Derwas N. and Poulos A. (1987). Very long chain fatty acid β-oxidation by rat liver mitochondria and peroxisomes. *Arch. Biochem. Biophys.*, **259**: 382-90.

Singh I., Moser H., Moser A. and Kishimoto Y. (1981). Andrenoleukodystrophy: Impaired oxidation of long chain fatty acids in cultured skin fibroblasts and adrenal cortex. *Biochem. Biophys. Res. Commun.*, **102**: 1223-9.

Singh I., Moser A., Goldfischer S. and Moser H. (1984). Lignoceric acid is oxidized in the peroxisome: Implications for the Zellweger cerebro-hepato-renal syndrome and andrenoleukodystrophy. *Proc. Natl. Acad. Sci. USA*, **81**: 4203-7.

Singh H. and Poulos A. (1988). Distinct long chain and very long chain fatty acyl-CoA synthetases in rat liver peroxisomes and microsomes. *Arch. Biochem. Biophys.*, **266**: 486-95.

Small G. (1993). Peroxisome biogenesis. Chapter 1 in Peroxisomes: Biology and Importance in Toxicology and Medicine. Ed. Gibson, G. and Lake, B. Taylor and Francis, Washington, DC.

Sorof S. and Custer R. (1987). Elevated expression and cell cycle deregulation of a mitosis-associated target polypeptide of a carcinogen in hyperplastic and malignant rat hepatocytes. *Cancer Res.*, **47**: 210-20.

Stott W. (1988). Chemically induced proliferation of peroxisomes: Implications for risk assessment. *Reg. Toxicol. Pharmacol.*, 8: 125-59.

Stryer L. (1995). Biochemistry - Fourth Edition. W.H. Freeman and Company, New York.

Stremmel W., Strohmeyer G. and Berk P. (1986). Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc. Natl. Acad. Sci. USA*, **83**: 3584-8.

Stremmel W., Strohmeyer G., Borchard F., Kochwa S. and Berk P. (1985). Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. *Proc. Natl. Acad. Sci. USA*, **82**: 4-8.

Suzuki H., Yamada J., Watanabe T. and Suga T. (1989). Compartmentation of dicarboxylic acid β-oxidation in rat liver: Importance of peroxisomes in the metabolism of dicarboxylic acids. *Biochim. Biophys. Acta*, **490**: 25-30.

Svoboda D., Grady H. and Azarnoff D. (1967). Microbodies in experimentally altered cells. *J. Cell Biol.*, **35**: 127-52.

Sweetser D., Heuckeroth R. and Gordon, J. (1987). The metabolic significance of mammalian fatty acid-binding proteins. Abundant proteins in search of a function. *Annu. Rev. Nutr.*, **7**: 337-59.

Thomas J., Debeer L., De Schepper P. and Mannaerts G. (1980). Factors influencing palmitoyl-CoA oxidation by rat liver peroxisomal fractions. Substrate concentration, organelle integrity and ATP. *Biochem. J.*, **190**: 485-94.

Thompson J., Winters N., Terwey D., Bratt J., Banasazak L. (1997). The crystal structure of the liver fatty acid-binding protein: A complex with two bound oleates. *J. Biol. Chem.*, **272 (11)**: 7140-50.

Thumser A., Evans C., Worrall A. and Wilton D. (1994a). Effect of ligand binding of arginine mutations in recombinant rat liver fatty acid-binding protein. *Biochem. J.*, **297**: 103-7.

Thumser A., Voysey J. and Wilton D. (1994b). The binding of lysophospholipids to rat liver fatty acid-binding protein and albumni. *Biochem. J.*, **301**: 801-6.

Thumser A., Voysey J. and Wilton D. (1996). Mutations of recombinant rat liver fatty acid-binding protein at residues 102 and 122 alter its structural integrity and affinity for physiological ligands. *Biochem. J.*, **314**: 943-9.

Thumser A. and Wilton D. (1994). Characterization of binding and structural properties of rat liver fatty-acid-binding protein using tryptophan mutants. *Biochem. J.*, **300**: 827-33.

Thumser A. and Wilton D. (1995). The binding of natural and fluorescent lysophospholipids to wild-type and mutant rat liver fatty acid-binding protein and albumin. *Biochem. J.*, **307**: 305-11.

Thumser A. and Wilton D. (1996). The binding of cholesterol and bile salts to recombinant rat liver fatty acid-binding protein. *Biochem. J.*, **320**: 729-33.

Tucker M. and Orton T. (1993). Toxicological studies in primates with three fibrates. Chapter 18 in Peroxisomes: Biology and Importance in Toxicology and Medicine. Ed. Gibson, G. and Lake, B. Taylor and Francis, Washington, DC.

Tugwod J., Issemann I., Anderson R., Bundell K., McPheat W. and Green S. (1992). The mouse peroxisome proliferator activated receptor recoginizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *Embo. J.*, **11**: 433-9.

Vanden Heuvel J. (1996). Perfluorodecanoic acid as a useful pharmacologic tool for the study of peroxisome proliferation. *Gen. Pharmac.*, **27 (7)**: 1123-9.

Van Rafelghem M., Vanden Huevel J., Menahan L. and Peterson R. (1988). Perfluorooctanoic acid and lipid metabolism in the rat. *Lipids*, **23**: 671-8.

Vianey-Liaud C., Divry P., Gregersen N. and Mathieu M. (1987). The inborn errors of mitochondrial fatty acid oxidation. *J. Inherit. Metab. Dis.*, **10** (Suppl. 1): 159-98.

Vincent S. and Muller-Eberhard U. (1985). A protein of the Z class of liver cytosolic proteins in the rat that preferentially binds heme. *J. Biol. Chem.*, **260**:14521-8.

Wallace K. (1998). Personal Correspondence. Experience in mitochondrial inhibition experimentation.

Wilkinson T. and Wilton D. (1986). Studies on fatty acid-binding proteins: The detection and quantification of the protein from rat liver by using a fluorescent fatty acid analogue. *Biochem. J.*, **238**: 419-24.

Zisman W. (1964). Relation of the equilibrium contact angle to liquid and solid constitution. Chapter 1 in Contact Angle Wettability and Adhesion- Advances in Chemistry Series 43. Ed. Gould R. American Chemical Society, Washington, D.C.