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STUDY OF EFFECTS OF FIBRATE DRUGS ON RAT FABP LEVELS/BLOOD
PRESSURE AND ISOLATION AND PURIFICATION OF FATTY ACID
BINDING PROTEIN FROM CHICKEN LIVER.

Thesis

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The Degree

Master of Science in Chemistry

By

Ravichand Yalamanchili

UNIVERSITY OF DAYTON

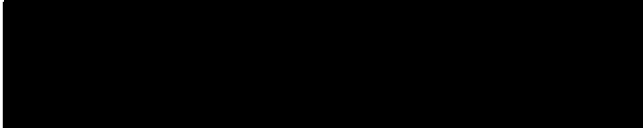
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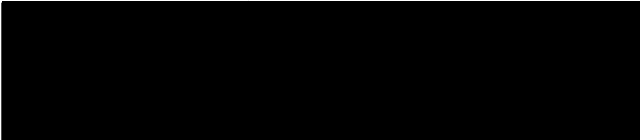
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ABSTRACT

STUDY OF EFFECTS OF FIBRATE DRUGS ON RAT FABP LEVELS/BLOOD PRESSURE AND ISOLATION AND PURIFICATION OF FATTY ACID BINDING PROTEIN FROM CHICKEN LIVER.

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Coronary heart disease and abnormal blood pressure are the biggest challenge to mankind in the modern world. Food habits and lack of exercise are the major causes of these problems. People who eat high fat diet are at more risk to get this disease and need more exercise. Our efforts in the laboratory mainly sought to prove that there a correlation between Fatty Acid Binding Protein (FABP) levels in the body and Blood pressure. FABPs are believed to play a major role in determining the levels of fatty acids in the body. The study of FABPs mainly focuses on the interaction of the heart disease drugs and their effects on the body. The two drugs which were tested on rats were fenofibrate, which was injected to female spontaneous hypertensive Rats (SHRs) and gemfibrozil, which was mixed in the diet and fed to Female Holtzman rats. The FABP levels and also the blood pressures of these rats were compared. The FABP levels were found to increase after the administration of these drugs.

The blood pressure of the SHR's was found to decrease after the administration of fenofibrate. The second objective was to use chicken liver to isolate FABP. Chicken liver was used to develop a cheaper alternative for FABP. The FABP isolated from chicken liver was purified via several chromatographic techniques. Finally chicken FABP was characterized using electrophoresis, isoelectric focusing, molecular weight determination and radioactive binding studies to compare the FABP obtained from chicken liver to that obtained from other tissues.

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TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENT.....	v
LIST OF ILLUSTRATIONS.....	viii
LIST OF TABLES.....	ix
LIST OF SYMBOLS/ABBREVIATIONS.....	x

CHAPTER

I. INTRODUCTION.....	1
II. MATERIALS AND METHODS.....	11
Materials.....	11
i Animals.....	11
ii Chicken liver tissues.....	12
iii Chemicals and Materials	12
iv. Solutions.....	13
Experimental methods with rats.....	14
i. Cytosol preparation.....	14
ii. Chromatographic methods.....	14
iii. Assay methods.....	15
iv. Calculations.....	16

Isolation and purification of chicken liver FABP.....	16
i. Liver supernatant preparation.....	16
ii. Ammonium sulphate cuts.....	16
iii. Chromatographic methods.....	17
iv. Assay methods.....	18
v. Characterization of chicken liver FABP.....	19
Molecular weight determination.....	19
Comparison of ability of chicken liver FABP to bind to rose Bengal, oleate, and palmitate.....	20
Electrophoresis.....	22
Isoelectric focusing.....	22
III Results and Discussion.....	25
i. Work done on rat tissue.....	25
ii. Work done on chicken liver tissue.....	27
IV Conclusions.....	36
V Future Directions.....	37
VI. References.....	38

LIST OF FIGURES

1. Protein and pink assay plot for Sephadex G-75 column (rat liver).....	26
2. Protein and pink assay plot of G-75 pool (chicken liver).....	29
3. Protein and pink assay plot A-50(chicken liver).....	30
4. Protein and pink assay plot of a G-50 chromatogram.....	31
5. Gel electrophoresis.....	35
6. Determination of the molecular weight of FABP.....	35
7. Comparison of affinity of binding of FABP to radioactive oleate and palmitate.....	36
8. Isoelectric focusing-appearance of two peaks.....	37

LIST OF TABLES

1. Solutions used for isoelectric focusing	23
2. Effects of fenofibrate and gemfibrozil on female rat FABP levels and LFABP/IFABP	26
3. The FABP content of the G-75 and A-50 pools	30
4. The FABP content of the A-50 and G-50 pools	32
5. Purification results of one of the six experiments (Trial #4)	32
6. Determination of molecular weight of FABP	34

LIST OF ABBREVIATIONS

ALBP.....	Adipocyte lipid binding protein.
AMS.....	Ammonium sulphate cuts.
BSA.....	Bovine serum albumin.
CRBP.....	Cellular retinoic acid binding protein.
CLO.....	Clofibrate.
CMC.....	Carboxy methyl cellulose
cDNA.....	Deoxyribonucleic acid.
DEAE.....	Diethylaminoethyl.
DI.....	De-Ionized water
DM.....	Direct Method.
ETOH.....	Ethyl alcohol.
FABP.....	Fatty acid binding protein
FEN.....	Fenofibrate
GEM.....	Gemfibrozil.
HFABP.....	Heart fatty acid binding protein
HPLC.....	High performance liquid chromatography.
Hoz.....	Holtzman Rats
IFABP.....	Intestinal Fatty acid binding protein.
IEF.....	Isoelectric focusing
IHM.....	In house method.
LFABP.....	Liver fatty acid binding protein
MDGI	Mammary derived growth inhibitor

NMR.....Nuclear magnetic resonance
PPAR..... Peroxisome proliferator-activated receptor
RBU..... Red-binding units.
SHR..... Spontaneous hypertensive rats.
TSM.....0.050M Trishydroxymethylamino-
methane, 0.25 M Sucrose,
3mM Mercaptoethanol (pH=7.5)

CHAPTER I

INTRODUCTION

Fatty acid-binding proteins are often referred to as intracellular lipid binding proteins as they play a very important role in absorption, transport and metabolism of free fatty acids within the cells. Unlike some proteins like albumins and globulins they do not transport proteins out side the cell there by separating them functionally from other extra cellular proteins. Originally these fatty acid-binding proteins were isolated by Ockner (1) from intestinal mucosa. These proteins were originally referred to as low molecular weight cytosolic proteins (1). Later immunochemical analysis based on reactivity to different antibodies was performed by Ockner and Manning (2-3).

These studies indicated the presence of different kinds of lipid binding proteins in different tissues. For example hepatic fatty acid binding proteins are not identical to intestinal fatty acid binding proteins. Therefore FABPs are named in accordance with the tissue where they are found. LFABP is probably the first identified in the group. Most commonly found lipid binding proteins include liver, intestinal and heart lipid-binding proteins (3). These types are differentiated based on difference in reactivity to different antibodies. Studies have also found that more than one type of lipid-binding protein may be present in same tissue for example hepatic and intestinal FABPs are present in jejunum (4-5).

Presently nine members of intracellular lipid-binding proteins have been isolated. They include Liver FABP, Heart FABP, Intestinal FABP, Adipocyte lipid binding protein (ALBP), Mammary derived growth inhibitor (MDGI), Myelin P2, cellular retinoic acid binding protein (CRBP1 and CRBP2). These proteins have been

isolated from various mammalian species. By now it is evident that intracellular transit of fatty acids is mediated by tissue specific protein carriers.

Primary structures of various FABPs have been determined by direct Edman degradation analysis (6-7). This protein sequence analysis revealed that although there is a wide variability of protein sequence there is much less variation in the overall protein structure. This clearly indicates that these proteins play a very important role in fatty acid trafficking based on the metabolic demands of the cells (7). The exact role of FABP in the body is still unclear, however it is believed to play an important role in intracellular and intercellular transport of free fatty acids across the phospholipids membrane (8).

The role of FABP in insulin sensitivity in connection with type-2 diabetes/obesity, mediating effects of peroxisome proliferators and cytoplasmic transport of hydrophobic molecules (e.g., 31) has been widely explored. In vitro and whole animal studies support FABP action in fatty acid oxidation (32) and action of cardiovascular drugs like clofibrate and other fibrates (33).

Because LFABP levels and transport capacity changes in early fatty liver development, it is seen as a rate limiting factor for liver lipid metabolism and related processes, as shown by LFABP build up due to hypolipidemic clofibrate, accompanied by increased fatty acid uptake (28). These FABPs have similar molecular masses and structures but different amino acid sequences. Varied FABP content in different organs/ organisms (e.g., much more in rat liver than intestine) may be related to ability to metabolize/transport lipids. Also, we found rat LFABP levels to drop on aging (34) and this may relate to obesity in old rats. All the differences support FABP acting in heart disease and species variation in nutrition capacity. Relationships of FABPs to heart disease, diabetes, and high body lipid

content in aging are probable. In heart disease, treatments with hypolipidemic drugs (e.g., clofibrate) may be due to raised LFABP levels.

FABP structure

The FABPs present in different tissues were found to be structurally and functionally distinct. Primary sequence information of various FABPs was obtained by various techniques such as Edman degradation analysis, cDNA, and genomic clones (7). Primary sequence studies of various members of the FABP family revealed that they show extensive similarity in their amino acid sequence. About 25 to 70% of amino acids in different FABPs were found to be identical although their surface charges were found to be considerably different (8). Later studies indicated that a particular protein isolated from same tissue of different species exhibited greater similarity in sequence than two different proteins isolated from different tissues in the same protein (11). Different types of FABPs occur in many rat tissues. Most studied are liver, heart and intestine. These FABPs, have similar molecular masses and structures but different amino acid sequences. Varied FABP content in different organs/ organisms (e.g., much more in rat liver than intestine) may be related to ability to metabolize/transport lipids.

These studies also revealed their molecular weights, which were found to vary from 14-15Kda. Their amino acid chain length varies from 127-133 amino acids. Some tissues like stomach and kidneys have different types of FABPs in different cell types, while others different FABPs in the same cells (e.g: enterocytes). Heart type FABP is the most universal one. Most of the FABPs bind to only one fatty acid molecule, except the liver type which binds to two free fatty acid molecules at a time. Most of the FABPs differ in primary structure, chromosomal localization of their

genes, regulation of their genes and the response of their concentration to changes to physiological and pharmacological conditions (14).

The basic structure of FABP consists of ten antiparallel beta strands which are joined by short reverse turns. The first two beta strands are connected by two alpha helices. The beta strands are organized to form two beta sheets. Each beta sheet constitutes half a "clam shell" with the strands in each half oriented in an orthogonal fashion to each other (16). It is believed this α -helical domain, along with the β C-D and D-E turns, functions as a "dynamic portal" that regulates FA entry and exit from the internal ligand binding cavity (46).

The conformation and binding of FABPs to ligands is influenced by difference in amino acid composition, volume distribution of binding pockets and solvent localization. The fatty acid assumes different conformations in different FABPs. The binding of FABPs to various fatty acids does not involve any ionic or covalent bonding; it may involve electrostatic interactions and hydrogen bonding with the carboxyl groups of fatty acids. NMR studies indicated that in liver FABP, fatty acids are closer to the surface than that in intestinal FABP (9).

The ligand specificity has been well established for liver and heart FABP. Liver FABP binds to a variety of hydrophobic ligands such as acyl-CoA, acylcarnitine, eicosanoids, lysophospholipids, haem, some steroid hormones, and peroxisome proliferators (11). IFABPs bind more readily to bile acids than to fatty acids (13). Recent studies have also shown that human liver and muscle FABPs have different affinities for fatty acids of different chain length different number and nature of double bonds (11). Stearic, oleic and elaidic acids are bound in the internal cavity of muscle FABP in the same arched conformation (16).

Functions of FABPs

The exact functions performed by FABPs are still unclear but some of the functions attributed to these proteins are supported by various studies that have been carried out on these tissues till now. It was believed for a long time that FABPs were involved in fatty acid trafficking across the cell membranes and within cells (15). Experiments conducted in the past have also revealed that these proteins help in fatty acid trafficking even between the cell organelles (14). It was found that FABP-bound fatty acids were well utilized by membrane bound organelles such as mitochondria for oxidation and microsomes for lipid synthesis (15). A correlation was found between FABP content and the fatty acid oxidation capacity of some rat tissues (15).

The endogenous lipid composition of liver FABP is strongly influenced by dietary fatty acid composition. It was found that rats which were fed with high fat diet have shown elevated levels of liver FABP, when compared to rats which were fed a diet with a low fatty acid content under same conditions (10). The content of FABP was found to increase by 50% in adipose tissue with a high fat diet (15). However in conditions such as starvation and streptozocin diabetes the FABP levels and FABP mRNA levels were found to be very low in rat adipose tissue (12).

The precise function(s) of FABPs remain poorly defined. More than 20 years ago, Tipping and Ketterer (39) proposed that soluble binding proteins may stimulate cytoplasmic transport of their ligands by increasing their aqueous solubility. FABP has been shown to stimulate intracellular fatty acid mobility in cultured cells (40), perfused rat liver (41), and artificial cytoplasm (42), whereas knockout of the gene for heart FABP was found to greatly impair cardiac fatty acid metabolism (43). Weisiger and co-workers (41) have shown that the rate of fatty acid diffusion within liver cells is directly proportional to the concentration of liver FABP, and they have proposed that

soluble binding proteins act as an aqueous carrier system that reduces the binding of fatty acids and other molecules to immobile cytoplasmic membranes (40).

On the other hand, Zucker and co-workers (44) found a decreased rate of fatty acid transfer between vesicles in the presence of a membrane-inactive binding protein (liver FABP), although a membrane-active binding protein (intestinal FABP) stimulated transfer under similar conditions (44). Certain tissues such as intestinal mucosa contain both membrane-active and membrane-inactive forms of FABP (44). Collectively, these findings suggest that the two classes of binding proteins may have distinct functions.

Interactions of FABPs

A major function for differentiated mammalian enterocytes and hepatocytes centers on their ability to direct intracellular trafficking of long chain fatty acids, both in connection with the processing of exogenous dietary lipid and in complex metabolic regulation of endogenous lipid homeostasis (13). Key to such regulation is ability to direct delivery of fatty acids to sites of complex lipid synthesis and to provide a physiological buffer in order to accommodate fluxes that accompany changes in fatty acid delivery. In regard to hepatic and intestinal fatty acid metabolism, there is an obvious need to accommodate large periodic fluxes, such as might occur in connection with dietary lipid ingestion or alternatively in association with changes in hepatic fatty acid uptake. An important component of such accommodation likely occurs following fatty acid uptake across the plasma membrane, where tissue-specific regulation of fatty acid binding has been attributed to distinct members of a large multigene family of small cytosolic lipid-binding proteins, among them liver (LFABP), heart (HFABP), adipocyte (AFABP), and intestinal fatty acid-binding protein (IFABP) (43). LFABP is expressed as an abundant gene product in differentiated

enterocytes and hepatocytes, with lower levels of expression in the kidney and colon (43). LFABP binds a broad range of ligands, including fatty acids, with a preference for unsaturated *versus* saturated fatty acids, branched-chain fatty acids, cholesterol, and bile acids (46).

Extensive study using *in vitro* binding and crystallographic analyses indicate that each molecule of LFABP binds two molecules of fatty acid (47), with binding facilitated through diffusional interactions (48). These data, coupled with studies from both transfected cells (49) and also from quantitative estimates of endogenous hepatic fatty acid binding activity (50), suggest that LFABP is likely the most important single source of such binding in mammalian liver.

In addition to fatty acid binding and sequestration, emerging evidence suggests that LFABP may be involved in regulating the activity of peroxisome proliferator-activated receptor (PPAR) through its ability to shuttle ligand (polyunsaturated fatty acids and peroxisome proliferators) to the nucleus (51). Further study has revealed that LFABP interacts physically with PPAR both *in vitro* and *in vivo*, providing a plausible basis for the possibility that ligand delivery via LFABP may represent a potential regulatory restriction point in this metabolic cascade process (51).

However, despite the observation that L-FABP represents one of the most abundant gene products in enterocytes and hepatocytes, details of its physiological functions *in vivo* have yet to be elucidated. Studies in HepG2 cells implicated LFABP in fatty acid uptake following antisense RNA expression (49), while gain of function experiments revealed increased fatty acid uptake and augmented lipoprotein secretion from rat hepatoma cells (52), suggesting that LFABP may play an important role in fatty acid delivery and metabolic utilization.

In order to study the role of LFABP in a more representative physiological context, a mouse line has been generated with a targeted deletion in the LFABP gene (39, 40). These mice were used to examine elements of the physiological adaptations associated with prolonged fasting, a well established model of altered hepatic fatty acid flux in which mobilization of adipose tissue triglyceride (TG) stores results in the release of large amounts of fatty acids for delivery and uptake by hepatocytes (40).

Basis for work on Rats

The basis for the work is as follows. LFABP and IFABP act in food fatty acid transport from intestine to blood and finally to liver where they are metabolized to produce energy and biosynthesize cell components (17-20). So, FABP gene lesions or variation of the ratio of LFABP/IFABP content could alter lipid uptake or metabolism or cause lipid disease. Our lab group has shown significant differences of LFABP and IFABP levels and low LFABP/IFABP in spontaneously hypertensive rats (SHR) and rat controls (21, 22). This implied raised blood lipid levels that may be related to atherosclerosis/hypertension. Related, significant changes of LFABP and IFABP levels and rises of LFABP/IFABP in Holtzman rats fed clofibrate (23, 24) fit with its prevention and reversal of cardiovascular problems by the drug. For better explanation of present data we began examining effects of other anticardiovascular drugs on LFABP and IFABP (e.g., fenofibrate [FEN], a more potent clofibrate cousin [fibrate]).

Our routine method for study of FABP levels, Sephadex G-75 chromatography and rose Bengal assay (25, 26, and 27) takes a whole day to study each rat. So, it would be valuable to develop an assay of rat FABP in cytosol which can be done in a few hours. We have had success here via a spectrofluorimetric method (DM)

developed by Mridul Bathula (38). Furthermore, rat liver is used for making FABP used in design of the method. Purchasing purified LFABP is expensive. Also, a rat liver weighs only 10-20 g, and many rats must be killed to make pure FABP by our UD method (28) which provides FABP for our work, still a lower price than the bought FABP. We thus seek alternatives to rat liver for making FABP and minimizing killing of the rats. Our preliminary work (38) with beef liver (by Brandon Dreyer) and pork liver (by Soujanya Varre) show that such inexpensive tissue, available via grocery stores may be useful for this purpose. Beef liver is a poor FABP source, and while pork liver is somewhat better, other sources (e.g., my work with chicken liver) needed to be investigated.

Significance

My research and its follow ups will add to our ability to study FABPs and to extend the understanding of LFABP and IFABP content and LFABP/IFABP ratio in hypertensive and normotensive rats first fed/intubated with fibrate drugs, support a basis for hypolipemic effects of fibrates and account for fibrate prevention and reversal of cardiovascular symptoms. Furthermore data will show whether FEN administration to 11-14 month old SHR lowers blood pressure and raises LFABP/IFABP more than clofibrate. The planned work has used the older rats to expand our rat-dog model for FABP actions, developed by Dr. Singer, beginning with great species differences of these factors in rats and beagles (21, 22). Because beagles have cardiovascular problems and rats are relatively resistant to such disease, the model was explored. Data taken in our lab with rats, thus far support the model.

- 1) LFABP/IFABP drops in SHR rats, compared to WKY controls.
- 2) CLO and FEN raise LFABP/IFABP in normotensive Holtzman rats.

3) CLO raises the LFABP/IFABP and lowers blood pressure in SHR of various ages.

4) In 4 month old SHR FEN have effects like CLO.

Planned work should support the submission of outside grant proposals. The "direct assay" should facilitate the ability of our group and others to clarify aspects of LFABP and IFABP actions and is expected to simplify uncovering information concerning FABP roles in health and disease to ultimately improve treatment of lipid-related human disease. Isolation of chicken liver FABP could yield an alternate, inexpensive source of FABP for researchers throughout the world.

Objectives

1) Study FEN effects on LFABP and IFABP levels, as well as the LFABP/IFABP ratio in 11-14 month SHR and Holtzman rats, to show its potential relation to lipid level alteration and blood pressure.

2) Develop a method for isolating FABP from chicken liver and partially characterize the isolated FABP.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Female Holtzman Sprague-Dawley rats, and female spontaneously hypertensive rats (SHRs), were purchased from Harlan Sprague-Dawley, Indianapolis, IN. They were maintained in the UD vivarium on a diet of Formulab Diet #5008 (PMI Nutrition International, Brentwood, MO) and tap water, ad libitum, until used. I collaborated in this work with Danielle Nyirandutiye. We used ~ 60 rats, divided into 10 groups of ~6 animals. 33 SHRs were either controls or intubated with either fenofibrate or gemfibrozil. 28 Holtzman rats were controls or intubated with gemfibrozil. We used a total of 30 females which include 12 Female Holtmans of which 8 are controls and 4 are injected animals. The remaining 18 were SHRs, which includes 8 controls and 10 injected animals.

The SHRs fed with diet were 11-14 months old. They were fed every other day and used 2-3 weeks after start of the experiment. The fenofibrate diet (Sigma, Saint Louis, MO) consisted of 25mg/ml drug (1 mL/100g BW, dose 2.50mg/kg per g in 1% CMC vs 1% CMC). The intubated Holtzman rats were also 11-14 months old. They were intubated every other day and used 2-3 weeks after intubation was started. The gemfibrozil injection consisted of 0.2% of the drug in 1% carboxymethylcellulose. UD's IACUC protocols 002-01 and 003-06 approved use of the animals for this work. They are on file in the office of the Vice President for Research and in the IACUC records.

Chicken liver tissues

Liver samples came from about 20 pounds of chicken liver purchased from supermarkets (Kroger and Meijer, Dayton, OH) as the work of Brandon Dreyer in our group (37) indicated that frozen beef liver samples sold for human consumption worked as well as fresh beef livers. We hoped to get better yields with chicken livers than those obtained with beef or pork liver, as chicken tissue is softer than pork liver. We believed that the ease of breaking the softer tissue would lead to higher yields, as pork liver has given higher yields (38) than harder beef liver (mechanical disruption of harder tissue could yield local heating effects that would inactivate FABP).

Chemicals and materials

The cardiovascular drugs fenofibrate and gemfibrozil, bovine serum albumin, chymotrypsin, human gamma globulin, horse myoglobin, ovalbumin and cytochrome-c, blue dextran, charcoal, rose Bengal, enzyme grade sucrose KCl and Tris base (trishydroxymethylaminomethane), were purchased from Sigma-Aldrich, (Saint. Louis, MO). Sephadex G-75, DEAE Sephadex A-50, Sephadex G-50 and Blue dextran 2000 were purchased from Pharmacia-LKB (Piscataway, NJ), Bio-Rad protein assay dye reagent came from Bio-Rad (Richmond, CA). [3H]Palmitate and [3H]Oleate (35.6 Ci/mmol) came from New England Nuclear corp.(Boston, MA). Phast gel gradient (10-15 % gels), Stain (Phast gel blue R solution), and buffer strips were from Amersham Biosciences (Uppsala, Sweden), Methanol, acetic acid, glycerol, sodium phosphate were products of Sigma (St Louis, MO). BioSafe-2 liquid scintillation cocktail was obtained from Research Products International (Mount Prospect, IL). All other chemicals and

materials used were obtained in the highest quality available from standard suppliers.

Solutions

The solutions used in these experiments include TSM buffer, Sephadex G-75 resin, rose Bengal dye, and dextran-charcoal. All solutions are prepared in deionized (DI) water, unless otherwise specified.

TSM. 86.5 g of sucrose, 6.05g Tris base and 0.03ml of 3M mercaptoethanol per liter of ice cold deionized water were combined, stirred, and titrated to pH 7.5. The mixture was stirred for 30 minutes in a cooler (4°C), and if needed, retitrated to ensure the proper solution pH (pH 7.5).

Rose-Bengal. 41mg rose-Bengal was mixed in 1L DI water for 10 minutes.

Dextran-coated charcoal. Na_2HPO_4 (0.028g) was dissolved in 200ml DI water and titrated to PH 7.4. One gram of dextran T-170 and carbon decolorizing Norit A (10g) were added and mixed for 3-5 days. Norit A and Dextran T-170 were purchased from Fischer Scientific (Cleveland, OH), and Na_2HPO_4 , purchased from Sigma chemical Co. (St. Louis, MO).

Control solution for injections. This solution was composed of 1% NaCl and 1% carboxymethyl cellulose (CMC) in deionized water.

Fenofibrate slurry. 25 mg/mL slurry was made from 1g of CMC and 2.5g fenofibrate added to each 100mL 1% saline solution and stirred for 60 minutes.

Gemfibrozil slurry. A 20 mg/mL slurry was made from 1g of CMC and 2.0g gemfibrozil added to each 100ml of 1% saline solution and stirred for 60 minutes.

1%NaCl. This was made by dissolving 10g NaCl in 1L deionized (DI) water. It was used to clean intestinal tissue.

Experiments with rats

Cytosol preparation

Rats were sacrificed by decapitation as approved by the UD IACUC. Blood pressures of the rats were taken on the night before sacrifice (25). Their livers and intestines were quickly removed, weighed and chilled. Then, ~1-2g liver portions were taken from each lobe of each liver used, and cut into small pieces. The first half of the intestine from each rat was trimmed free of adipose tissue, cleaned using saline, and weighed. From this point on, all preparative procedures were carried out at 0-4 °C. The liver and intestine samples were homogenized (25) separately in a Potter-Elvehjem homogenizer (27). In the case of intestine, muscle tissue was removed from homogenates, weighed, and its weight was subtracted from the weight of the intestine, to give mucosa weight.

Homogenates were prepared in 1mL/g tissue of ice cold TSM buffer. The homogenates were poured into centrifuge tubes, placed into a fixed angle 40 degrees in the rotor (Beckman, Fullerton, CA). Then the samples were centrifuged at 105,000xg for 60 min at 2° temperature and 1 torr pressure (Beckman L-5-65 Centrifuge, Beckman, Fullerton, CA) at 1°C within 15 minutes after preparation (27). Finally, the supernatants were drawn off and their volumes were recorded.

Chromatographic methods

Sephadex G-75 chromatography. The 3-5 ml samples from the preceding step were subjected to chromatography on the molecular sieve, Sephadex G-75, using 1.25x70 cm columns, filled with the molecular sieve (25). In every experiment, fractionation on G-75 columns was carried out simultaneously with samples from liver and intestine. The columns were pre-equilibrated with TSM and then elution with TSM was carried out. Sixty samples (1.5 ml each, fractions)

were collected, using a Redifrac fraction collector (Pharmacia LKB, Piscataway, NJ).

Assays methods

The fractions underwent three assays (25): 280nm protein assay to determine the location of non-FABP proteins; 560 nm rose Bengal assay to identify FABP rich fractions; a 560 nm pool assay to identify the amount of LFABP, IFABP and LAFABP/IFABP per g tissue and per 100g bodyweight.

280 nm protein assay. A 0.10 ml sample of each fraction chosen was mixed with 0.90 ml TSM and 280 nm absorbances were read (27) in a Genesys spectrophotometer (Spectronic instruments, Rochester, NY).

560nm rose Bengal assay. Samples (from every third chromatogram fraction) were mixed with TSM (intestine samples used 0.30 mL while liver samples used 0.10 mL of the sample). Next, 0.30 mL rose Bengal was added to each sample, followed by incubation (5 min, 0°C). Then, 0.15 mL charcoal-dextran was added, followed by incubation (8 min, 0°C). The tubes were then centrifuged for 8 minutes in a Centra B-Plus centrifuge (International Equipment Co., Needham Heights, MA) and their 560 nm absorbances were read. The 560 nm absorbances were plotted to identify the FABP-rich fractions.

560 nm pool assay. FABP rich fractions were identified, pooled, and their volumes were measured. They were diluted if necessary and the 560nm rose Bengal assay was carried out on each pool. Absorbances observed at 560 nm were converted into mg FABP per g liver and per 100 g body weight (RBU/ml).

Calculations

0.67 x RBU/mL	1.180
Total RBU (0.67 RBU/mL x pool vol)	23.6
mg FABP/g [Total RBU/(3.69 x corr. Weight)]	1.54
mg FABP/100g BW (mg FABP/g x TW/BW in 100s)	4.80

Statistical significance of differences between control and experimental groups ($p < 0.05$) was determined by Student's test (25, 27).

Isolation and purification of chicken liver FABP

Liver supernatant preparation

About 200 g of chicken liver was cut into small pieces, mixed with TSM (1mL for every g) and dispersed in a Waring blender for ~1 min, followed by homogenization in a Potter-Elvehjem homogenizer for 6 passes. All methods were carried out at ice bucket temperature. Next, the homogenate was poured into centrifuge tubes and centrifuged at 12,000 rpm in a GSA rotor (Dupont-Sorvall, Newton, Connecticut) for 40 minutes, using a Sorvall Superspeed RC2-B Centrifuge (Dupont-Sorvall, Newton, Connecticut). The supernatants were drawn off after centrifugation and total supernatant volume was recorded.

Ammonium sulfate (AMS) cuts

The supernatant was then subjected to consecutive AMS cuts {0-35%, 35-50%, 50 -92.5%}. This step separated chicken liver FABP from chicken liver in the 50-92.5% saturated AMS fraction. For each cut; the amount of solid ammonium sulfate to be added was calculated, depending on the volume of the supernatant. The ammonium sulfate was then weighed, triturated to a fine powder using a mortar and pestle, and added to the supernatant over 15 min with gentle stirring in an ice bath on a magnetic stirrer.

Then the sample was allowed to stir for an additional 10 min. Next it was centrifuged in the Sorvall centrifuge (SS-34 rotor) for 30 min at 30000 RPM. After centrifugation the pellets from the 0-35% and 35-50% cuts, which did not contain FABP, were discarded, while the pellet from the 50-92.5% cut --which held the FABP-- was resuspended in enough TSM to bring the volume of the resuspendate to 12-15 ml.

Chromatographic methods

DEAE Sephadex G-75 chromatography.

This work uses the resuspendates from the previous step and Sephadex G-75 columns (2.5 cm x 70 cm), eluted with ~500mL TSM buffer at a rate of 10 drops per minute. The resuspendates from 30-50% cut and 50-92.5% cut were loaded on separate columns and were eluted with TSM at a rate of 10 drops per minute. Fractions of 140 drops each were collected on a Redifrac fraction collector. After chromatography, fractions were chosen for protein (A-280) and FABP (A-560) assays. Protein assays used 10 fold diluted material. FABP assays used 0.30 mL of undiluted material. It was determined that the resuspendate from the 35-50% cut held no FABP and that from the 50-92.5% cut held the FABP.

DEAE Sephadex A-50 chromatography.

The pool from each G-75 step was applied to a DEAE Sephadex A-50 column (2 x 24-26 cm). The column was prewashed with 400 mL TSM and eluted with TSM in 80 drop fractions at 15 drops per min. Fractions were assayed at 280 nm and by the 560 nm, red charcoal method. FABP-containing fractions were pooled and pool volume was measured. Pools were reassayed by red charcoal assay, labeled, and frozen. A gradient maker was used with 400 mL

each of TSM and TSM- 0.35 M KCl. Fractions of 264 drops each were collected on a Pharmacia Redifrac fraction collector. The 280nm assay used chosen undiluted fractions. The 560 nm assay used 0.30 mL samples.

DEAE Sephadex G-50 step.

The pool from the A-50 step was applied to a Sephadex G-50 column (2.5 cm x 70 cm). The column was prewashed with 500 mL TSM and eluted with TSM at 5 drops per min. Fractions of 150 drops each were collected on a Pharmacia Redifrac fraction collector. Fractions were assayed at 280 nm and by the 560 nm, red charcoal method. FABP-containing fractions were pooled and pool volume was measured. Pools were reassayed by red charcoal assay, labeled, and then frozen. The 280nm assay used chosen undiluted fractions. The 560 nm assay used 0.30 mL samples.

Assay Methods

Biuret assay.

Ammonium sulfate cuts were diluted 1:20 (samples assayed 0.30 & 0.60 mL), G-75 pool 1: 4 (samples assayed 0.40 & 0.80 mL), A-50 pool 1:3 (dilutions 0.40 & 0.80 mL), G-50 pool:1:2 (samples assayed 0.40 & 0.80 mL) and BSA 5mg/ml (sample assayed 0.50 mL) with water. Four ml of biuret reagent was added to each tube, allowed to stand for 30 minutes and the tubes were read at 540 nm.

Coomassie blue assay.

Ammonium sulphate cuts were diluted 180 fold, G-75 pool was diluted 90 fold, A-50 pool was diluted 10 fold and G-50 pool was diluted 2 fold. Sample sizes of 0.10mL, 0.20 mL and 0.30 mL of each pool were taken in 12 different

test tubes. 0.50 mL of 5.0 mg/mL sample of bovine serum albumin was taken in 13th tube which is used as a reference. 14th tube, which does not have any protein, acts as a blank. The volume in each tube was made up to 1.00 ml by addition of TSM buffer. Finally 1.00 ml of Coomassie blue reagent (Bio-Rad, Fullerton, CA) and 3.00 mL of water were added and incubated for 15 minutes. Their absorbances were then measured at 595nm.

Conductivity tests.

Tubes 5, 10, 14, 21, 30, 35, 40, 45 and 50 were taken from the DEAE Sephadex A-50 chromatogram and their conductivities were measured on a Wheatstone bridge (Leeds & Northrup Co., Philadelphia, PA). The conductivities were plotted against the fraction numbers.

Characterization of chicken liver FABP

Molecular weight determination.

Before carrying out each experiment, 0.40 mL of the FABP pool saved from the G-50 column, was diluted 1 + 4 with TSM and assayed at 560 nm to re-check its activity. The pool's activity was found to be almost equal to the previous data and thus, usable. Marker proteins, 30 ml, of 1.5 mg/mL (i.e. 45mg protein for 30 ml TSM) of ovalbumin, myoglobin and cytochrome-c were also prepared. Samples of FABP pool (~8 mL) were each loaded on a G-50 column 1.9 cm in diameter and 70 cm in length. Then, 65 fractions of 75 drops each (10mL) were collected on a Redifrac fraction collector and samples were read at 280 nm.

The same amounts of as FABP of blue dextran, ovalbumin, cytochrome-c, myoglobin and chymotrypsin were loaded onto the column one after the other and 65 fractions of 75 drops each were collected. The fractions of blue dextran were read at 620 nm and marker protein fractions were read at 280 nm. Blue

Preliminary G-50 Tests.

0.5 ml of 1.16 mM chosen [^3H]fatty acid stock was diluted with 4.5 mL of 50 mM phosphate buffer. The diluted stock (16 μM) was made in a plastic test tube and discarded at day's end. To each plastic assay tube, 1.00 mL of a mixture of 49.2 mL of phosphate buffer plus 0.80 mL diluted stock (palmitate or oleate) 0.10 mL, chosen G-50 concentrate were added. This gave 1.93 mM oleate or palmitate in reaction mixtures. The tubes were mixed and allowed to stand 20 minutes at room temp. Then, 0.10 mL charcoal-dextran was added, mixed, and allowed to stand for 15 minutes at room temperature. The tubes were centrifuged for 10 minutes and 500 μL was taken to count. A known sample that contained 50 μL of diluted fatty acid stock was counted, for comparison. The fractions were quick counted for 1 min each then, recounted for 2 min. One sample of each G-50 concentrate and one sample of TSM as a blank were assayed (with 6 G-50 concentrates a total of 7 samples were assayed). Oleate fractions were quick counted for 1 min, then counted for 2 min with palmitate.

Column Fraction Assay.

Each G-50 column was assayed concurrently with the A-280 assay and the pink assay. Four experiments were carried out with each FA. Here, 0.05 ml of 1.16 mM chosen [^3H] fatty acid stock was diluted with 4.5 mL of 50 mM phosphate buffer. The diluted stock (16mM) was made in a plastic test tube and discarded at day's end. To each special plastic assay tube: (palmitate or oleate).0.40 mL, each column fraction were added. This gave 1.93 M oleate in reaction mixture. The tubes were mixed and allowed to stand 25 min at room temp. 0.10 mL charcoal-dextran was added and mixed, and allowed to stand for 25 min. The tubes were centrifuged for 5 min and 500 μL were taken to count. A

known sample that contained one of the diluted fatty acid stock was compared. The fractions were quick counted for 1 min each then, counted for 2 min. The process was repeated with palmitate.

Electrophoresis.

Phast electrophoresis (Pharmacia-LKB, Piscataway, NJ) was carried out on phast gel gradient 10-15% gels as described by the company (27). The media used were 1) Stain (0.1 phast gel blue solution in 30% methanol & 10% acetic acid in distilled water; 2) Destain (30% methanol and 10% acetic acid in D.I water) 3) Preserving solution (10% glycerol and 10% acetic acid in D.I water). BSA and rat FABP were used as standards.

The gel compartment was prepared by positioning the gel in the proper alignment in the compartment and placing a phast gel buffer holder with 2 buffer strips onto the separation bed. The sample applicator was loaded with BSA, AMS Cut, G-75, A-50, G-50 and rat FABP in separate lanes. Prior to application, the samples had been mixed with a blue tracking dye solution and denatured by boiling for 5 minutes. Separation was performed, followed by insertion of gels into the development chamber of the instrument and a development run was performed. The gels were then allowed to dry and were scanned.

Isoelectric focusing

This procedure is carried out by a method provided by the maker of the instrument, Pharmacia LKB (25). A 2.5 mL ample of a 40% solution of Ampholines (Pharmacia-LKB, Piscataway, NJ), was diluted with deionized (DI) water to 10 mL (Solution A). From this solution *dense solution* (7.5 mL of Solution A + 2.5mL of DI water + 28g of sucrose); less dense solution (2.5mL of Solution

A + 60 mL of DI water); *Anode solution* (0.2 mL of concentrated H_3PO_4 + 14 mL DI water +12g sucrose) and *Cathode solution* (0.2 mL ethylenediamine + 10mL DI water) were prepared. An isoelectric focusing column # 8100 (Pharmacia LKB, Piscataway, NJ), was prepared by filling the column with anode solution through the bottom by port #1. Twenty-four mixed ampholine solutions (Table 1) were then added through port #2, making sure that ampholine solution does not touch the cathode, a loop inside the fritted glass portion. The FABP (1.00 mL/tube) is added to tubes 10,11,12

Table 1: Solutions Used For Isoelectric Focusing

TUBE	DENSE SOLUTION (ML)	LIGHT SOLUTION (ML)
1	4.6	
2	4.4	0.2
3	4.2	0.4
4	4.0	0.6
5	3.8	0.8
6	3.6	1.0
7	3.4	1.2
8	3.2	1.4
9	3.0	1.6
10	2.8	1.8
11	2.6	2.0
12	2.4	2.2
13	2.2	2.4
14	2.0	2.6
15	1.8	2.8
16	1.6	3.0
17	1.4	3.2
18	1.2	3.4
19	1.0	3.6
20	0.8	3.8
21	0.6	4.0
22	0.4	4.2
23	0.2	4.4
24		4.6

Next, the column is filled to about 1cm above the cathode, with cathode solution. The electrodes are then attached and the power supply to which they are connected (Eps600, Pharmacia-LKB, Piscataway, NJ) is turned on at 100 volts overnight (~14 hours). Then it is turned up to 300 volts for 77 hours, until

an amperage of 0.5 mAmp is reached. Next, 80 drop fractions (~3.2ml) were collected in a Redifrac (Pharmacia-LKB, Piscataway, NJ) fraction collector at a rate of 6 drops per minute. Fractions were assayed for protein and FABP as already described. Their pHs were also read.

CHAPTER III

RESULTS AND DISCUSSIONS

Rat Tissue

These trials were carried out on livers and intestines of 11-14 month old female spontaneously hypertensive rats (SHRs) and female Holtzman rats (Hoz).

1) I explored fatty acid binding protein (FABP) levels in female SHR and Hoz plus or minus FEN, in collaboration with Danielle Nyirandutiye, to help us determine whether FABP level or ratio and blood pressure alteration was more extensive in the older groups than in the ~4 month group tested by others in our lab.

2) I compared levels of FABPs, LFABP/IFABP, and blood pressure, in SHR controls, SHRs given the test drugs gemfibrozil (GEM) and fenofibrate (FEN), to extend data already obtained by others in the group with male animals and other fibrates and also to compare the differences in the actions of GEM and FEN.

Work done on rats

SHR fenofibrate injection study.

For the first objective 33 SHR rats, divided into 4 groups of ~8 animals. SHR's, were either controls or rats injected with FEN. They were used in vivo and in vitro study of LFABP column chromatographic method described in previous sections of this report (e.g., Figure 1).

Holtzman Gemfibrozil Diet Study.

For the intubation study 28 Holtzman rats were used, divided into 4 groups of ~8 animals. Holtzman rats were controls or fed with gemfibrozil. They were

used in vivo and in vitro study of LFABP column chromatographic method described in previous sections of this report

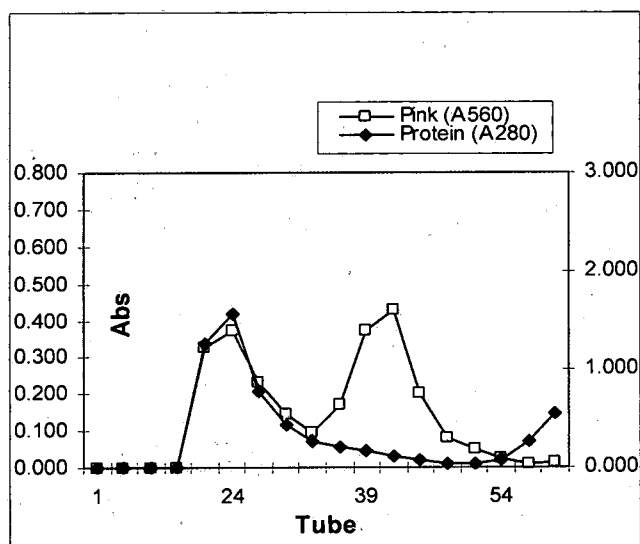


Figure 1: Protein and pink assay plot for Sephadex G-75 column

TABLE 2: Effects of FEN and GEM on female rat [FABP] and LFABP/IFABP.

*Statistical significance between experimental and control groups ($P < 0.05$)

Measured Quantity/g tissue	SHR Injected		Hoz -Fed with Diet	
	FEN(10)	CONTROLS(8)	GEM(4)	CONTROLS(8)
LFABP	3.54± 0.20	1.18±0.081	1.47± 0.14	1.15± 0.041
IFABP	1.17±0.060	0.670±0.027	0.459±0.034	0.453± 0.031
LFABP/IFABP	3.03±0.21*	1.82± 0.013	3.0±0.55	2.54± 0.021

SHR fenofibrate study

The [LFABP]/g tissue, shown in Table 1 and per 100g body weight (not shown) increased significantly after the FEN diet. IFABP increased to a smaller extent in the rats tested. A significant increase in LFABP/IFABP per g liver and

per 100g body weight also occurred in the animals given fenofibrate. The blood pressures in the treated SHR rats decreased significantly by ~24 mm Hg, as expected.

Holtzman gemfibrozil study.

[LFABP] per gram tissue, shown in Table 1, and per 100g body weight (not shown) increased significantly after gemfibrozil diet. IFABP did not increase significantly in either male or female animals. A significant increase in LFABP/IFABP per 100g body weight occurred in the male animals given gemfibrozil, while there was a smaller increase in ratio for females that was of little significance.

Work done on chicken liver

Ammonium sulfate cuts.

The aim of the initial ammonium sulfate (AMS) cut (0-50%) was to precipitate undesired tissue proteins present in the original homogenate leaving behind a sample enriched for FABP. The enriched FABP is separated from the solid precipitate by centrifugation.

The resulting supernatant was then subjected to a final AMS cut (50-92%), where the FABP is precipitated leaving behind a supernatant which does not contain FABP. This supernatant is separated by centrifugation and is discarded. The remaining precipitate which contains more enriched FABP is dissolved in TSM buffer. In one of the six preparations carried out (Trial 4), the homogenate volume was 450 mL. The first AMS Cut (0-50%) resulted in a decreased volume of 136 mL. The dissolved pellet from the 50-92 % cut was 40 mL. A large amount of extraneous protein was removed, leaving behind a higher

concentration of FABP/ml than present in the original homogenate. This final dissolved pellet was loaded onto the G-75 column.

G-75 chromatography.

For the initial experiments 50 g of chicken liver was mixed with TSM and blended .The homogenate produced was subjected to centrifugation to separate the cytosol . The cytosol was then subjected to a variety of AMS cuts (0-35%, 35-50%, 50-75%, 50-80%, 50-90% and 50-92%).Then the cytosol was loaded onto a G-75 column which was prewashed with 150 mL of TSM and the fractions were collected. The fractions collected were then analyzed. Protein (280nm) and pink assays (560nm) were performed and the FABP was pooled. Finally the pool was subjected to pool assay at 560 nm. Maximum yield of FABP was obtained for 50-92% cut. The tissue was scaled up from 50g to 100g and finally to 200g and then protein and pink assays were performed on the G-75 fractions. The absorbance data were graphed versus fraction number (Figure 2).

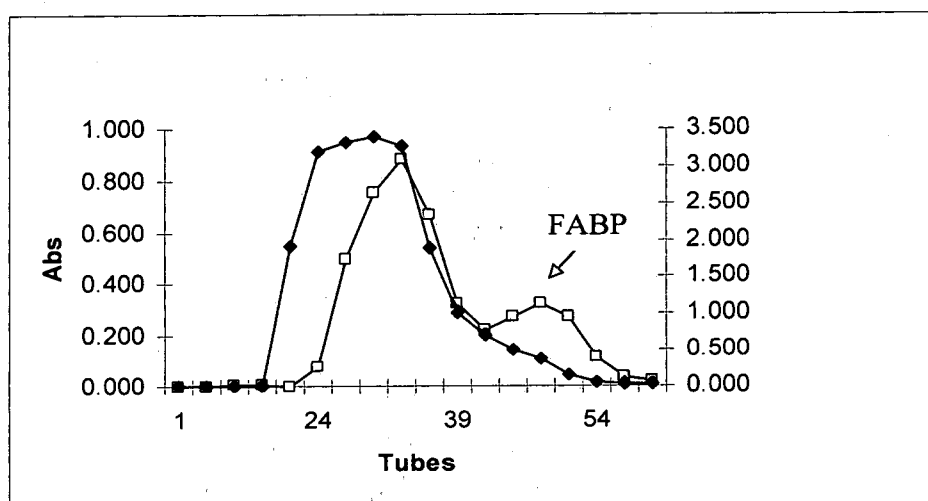


Figure 2: Protein and pink assay plot of G-75 pool

The two prominent A-560 peaks represent rose Bengal binding. The second, broader peak indicates where the FABP is located in the G-75 fractions. In the chromatogram shown in the figure, fractions 39-57 contain FABP. The pool collected had a volume of 115 mL. Five mL was saved for a pool assay; the rest of the pool (110 ml) was loaded on a DEAE Sephadex A-50 column.

DEAE Sephadex A-50 (A-50) step.

This step was repeated in 6 times. Protein and pink assays were again performed on the A-50 fractions. The absorbance data were graphed versus fraction number (as in Figure 3). The pink assay data showed one prominent peak, indicating where the FABP is located. In the experiment shown the fractions containing FABP were numbers 26-37. The pool volume was 103 mL. Since 103 mL is too large a volume to put on the Sephadex G-50 column used next, it was concentrated to 10 mL in an Amicon concentrator (see Methods).

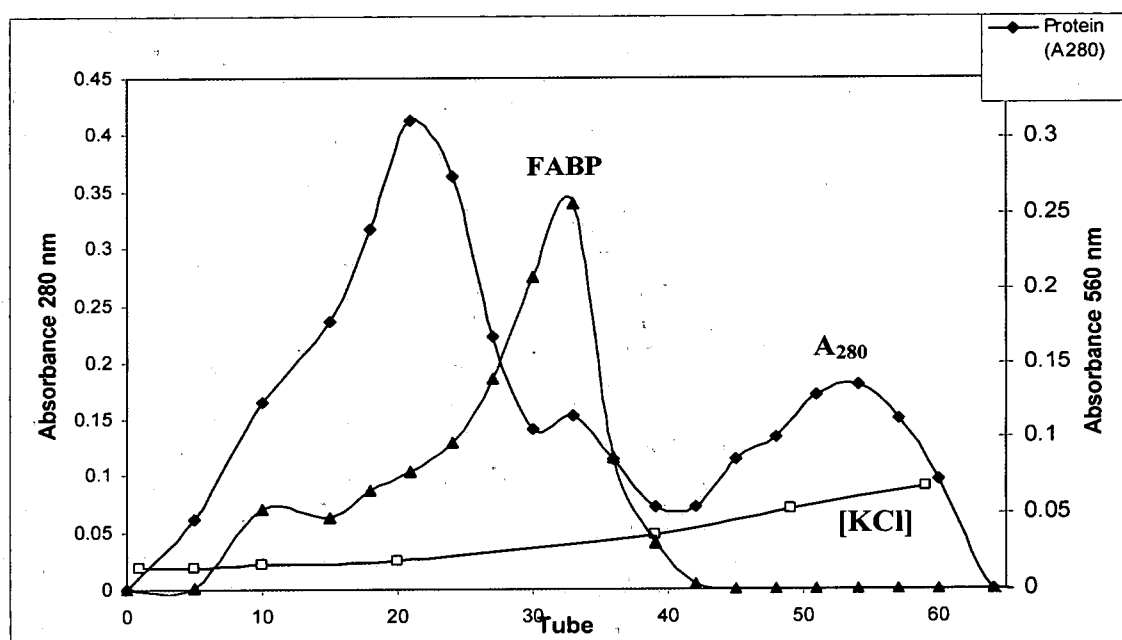


Figure 3: Protein and pink assay plot of an A-50 chromatogram

A comparison between the G-75 and A-50 results was made to determine the effectiveness of FABP recovery between the two steps. The Total Red b RBU was used to compare the steps (Table 3) this showed how much FABP was recovered between each step ($\% \text{ Recovery} = (A-50/G-75) = (45.1/80.7) = 55.9\%$).

Table 3: The FABP content of the G-75 and A-50 pools

	G-75	A-50
Total RBU	80.7	45.1

Sephadex G-50 (G-50) step.

The pool from the A-50 column was loaded on a G-50 column, prewashed with TSM. The fractions collected were analyzed at 280nm and 560nm. The absorbance data were graphed versus fraction number (e.g., Figure 4). As shown, there was one peak which contained FABP. The FABP-containing

fractions from this experiment were 18-33. Next, the FABP containing fractions were pooled and analyzed at 560 nm (Pool assay). The pool volume was 78 mL.

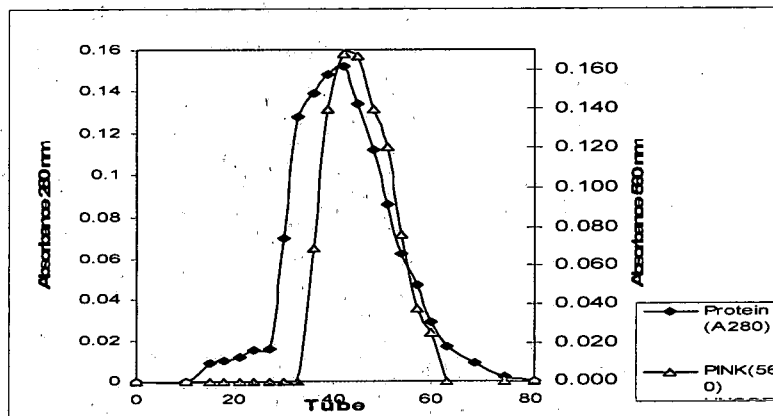


Figure 4: Protein and pink assay plot of a G-50 chromatogram

Comparison between the A-50 and G-50 results was made to determine the FABP recovery between the two step (**% Recovery = (G-50/A-50) = (32.4/45.1) = 71.8%**)

Table 4: The FABP content of the A-50 and G-50 pools

Pool	A-50	G-50
Total RBU	45.1	32.4

The amount of FABP recovered between each step was good and improved with each step. This is evidenced by the more than 75% recovery between the last two chromatography steps.

Total purification.

A progressive increase in % recovery of FABP supports the effectiveness of the successive chromatography method. The results of the biuret assay of pools were compared with the FABP (Pink) assay results to show the actual purification of FABP compared with total protein content (Table 7), as denoted by increased specific activity (SA).

Table 5: Purification results of one of the six experiments (Trial #4)

	AS Cut	G-75	A-50	G-50
(A)Avg. Pink Abs	456	26	3.85	5.09
(B)Avg. Biuret Abs	20064	2964	489	524
SA (A/B)	0.0038	0.0404	0.092	0.062
Fold Purification		10.63	2.27	0.674
Total Purification	10.63 x 2.27 x 0.674			16.26

The SA (specific activity) of FABP increased between each step except the last one. The fold purification (purification between each step) was determined by dividing the SA of the particular step by the SA of the previous step [e.g., (SA G-

75/SA AS Cut) = $(0.0404/0.0038) = 10.63$. The total purification found was 16.26.

The expected 12-fold purification from homogenate (27) must be taken into account. Therefore, the total estimated purification is $(16.26 \times 12) = 195$ Fold.

Thus, the FABP was probably purified 191 ± 5.0 , in the 6 preparations carried out.

Characterization of the purified FABP.

The most purified FABP was subjected to various techniques to determine its purity and characteristics such as molecular weight, binding characters etc. Isoelectric focusing was carried out to determine whether the FABP obtained has one or more isomeric forms. These characterization studies helped in identifying the purity and properties of chicken liver FABP. They also enabled me to compare differences between chicken liver FABP that from other sources.

Gel electrophoresis.

The gel was used to compare the purification of the sample FABP to purified AMS Cut, G-75, A-50, G-50, rat FABP, and BS in separate lanes. The results suggested that a single FABP was isolated. The electrophoretogram shows (Figure 5) that the final FABP obtained is more pure than that in the earlier ones.

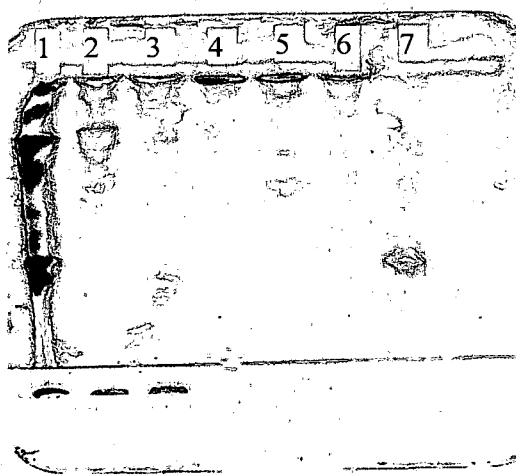


Figure 5: Gel electrophoresis. The numbers 1-7 represent AMS Cut, G-75, A-50, G-50, rat FABP, BS

Determination of the molecular weight of the FABP.

The molecular weight of the final FABP was determined by Sephadex G-50 chromatography of 10 mL of the G-50 pool. The same volumes of blue dextran, ovalbumin, cytochrome-C, myoglobin and chymotrypsin were loaded onto the column one after the other and 65 fractions of 75 drops were collected in each case. The fractions of blue dextran were read at 620 nm and the rest of the protein fractions at 280 nm. Table 8 shows the data collected for one of 6 experiments (Trial # 2). These data were used to plot a graph (Figure 6) relating the elution maximum for each marker protein, versus its molecular weight (\square).

Table 6: Determination of the molecular weight of the FABP

PROTEIN	Ve	MW	Ve/Vo	Log MW
Ovalbumin	31	40000	1.03	4.60
Myoglobin	41	17500	1.37	4.24
Cytochrome-c	43.5	12000	1.45	4.08
Chymotrypsin	35	25000	1.17	4.40
Blue dextran	30		1.00	
FABP	44	12100	1.47	4.08

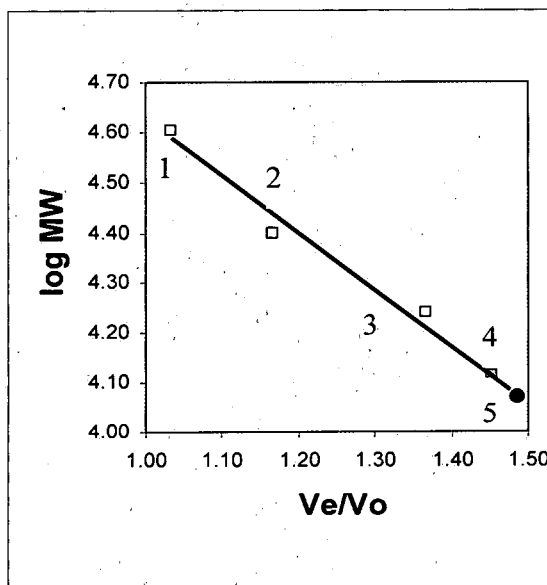


Figure 6: Determination of the molecular weight of FABP. The numbers 1-5 indicate respectively ovalbumin, chymotrypsin, myoglobin, cytochrome-c and FABP.

The molecular weight of the FABP (●) in the experiment shown was found to be 12.1 Kda. The average molecular weight of the FABP from the six experiments carried out was 12.2 ± 3.7 Kda

Comparison of the ability of chicken liver FABP to bind to rose Bengal, oleate and palmitate .

The ~200-fold purified FABP pools from chicken liver were taken, concentrated to ~4 mL and applied to a Sephadex G-50 column (1.95x70 cm) which was eluted at 8 drops/min. Ninety drop fractions were collected. A₂₈₀ readings were taken, rose Bengal & ³H-oleate/palmitate assays were performed and the data were compared. Figure 7 shows one of three experiments which gave similar results. From the graph the radioactive binding studies clearly indicate that FABP binds to oleate more than palmitate. Also, it appears that the preparation may contain 2 FABP species.

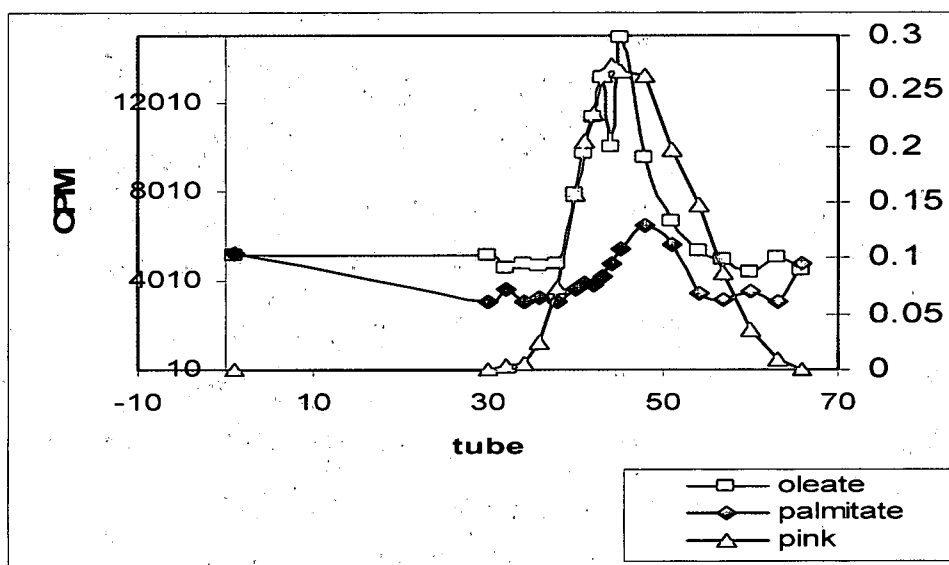


Figure 7: Comparison of the binding of FABP to rose Bengal, oleate and palmitate

Isoelectric Focusing (IEF).

A pink assay was performed on the IEF fractions. The 560 nm absorbance data and pH data were graphed versus the fraction number (Figure 8). The pink assay results show two different A-560 peaks which represent two types of FABP isolated. The main peak (Peak 1) was at pH 5.8 ± 0.02 and comprised a FABP recovery of 55 ± 0.50 %. Peak 2 was at a pH of 4.0 ± 0.03 and a % recovery of 22.6 ± 0.43 %. The total % FABP recovery was 77.6 ± 0.93 %. The unlabeled earliest peak (Peak 0) was not pink and may be due to refractance or turbidity. A significant amount of FABP was purified and isolated. However, the FABP sample was not homogenous since two types of FABP were present. The IEF data are similar to study results by others (27). Peak 1 and 2 indicates the presence of two Isomeric form of FABP.

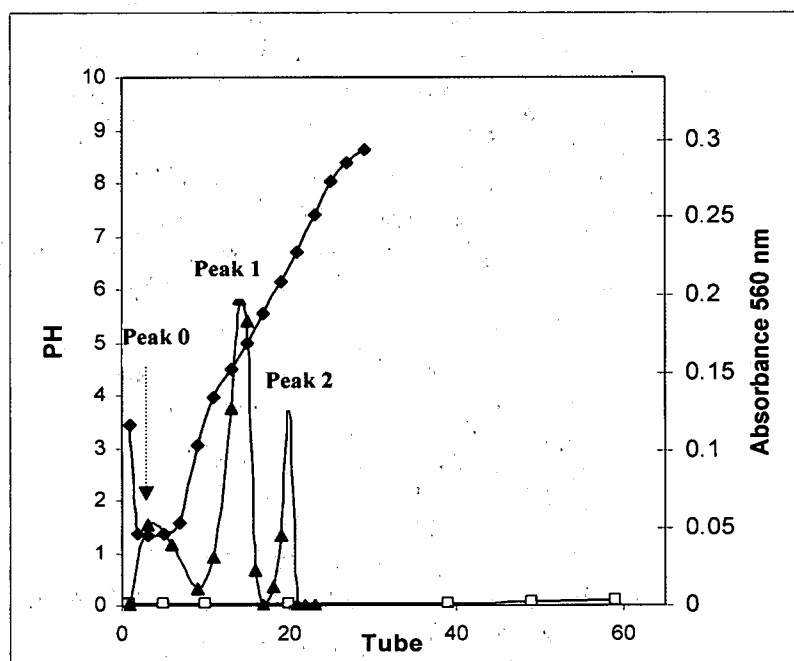


Figure 8: Isoelectric focusing-appearance of two peaks

CHAPTER IV

CONCLUSIONS

The purposes of this research were to study the drug effects on rat FABPs and to prepare purified, inexpensive chicken liver FABP, to characterize the chicken liver FABP compared to rat liver FABP, and to observe any differences between fresh rat tissue and frozen chicken tissue isolation yield. The experiment achieved its purposes. The experiments with rats showed a significant increase in both LFABP and IFABP in female hypertensive rats treated with fenofibrate. There was also an increase in LFABP/IFABP in female hypertensive rats treated with fenofibrate. The fenofibrate administered rats showed a sharp decrease in blood pressure that may have been due to the increase in FABP levels which indicates that fenofibrate increases the FABP levels in both liver and intestine. As for the preliminary study of gemfibrozil, a slight increase in LFABP and IFABP was observed in female Holtzman rats. Both fenofibrate and gemfibrozil studies concurred with the results of previous studies.

A useful, inexpensive FABP preparation was made from the chicken liver. However, the radioisotope and IEF results indicated, that a mixture of two FABPs were isolated. Therefore, the sample FABP was not homogenous. However, a comparison between rat and chicken liver FABP isolation yield was made. About 1/5 as much FABP/g was isolated from the chicken liver as compared to rat liver (21). My data is also similar to data reported from fresh pork liver (21). Therefore, there are similar results between fresh rat tissue and frozen chicken tissue FABP isolation.

CHAPTER V

FURTHER EXPERIMENTS

The current rat studies gave conclusive evidence that with a decrease in blood pressure there is a significant increase in FABP levels. Further quantitative studies can be carried out to correlate the effects of fibrates on HDL and LDL cholesterol levels, changes in body weight. In addition the effects of other cardiac drugs can be studied on the FABP levels in various tissues and also the FABP studies can be carried out to the next level (e.g., monkeys and humans) which may provide a significant break through in cardiac research.

A homogenous sample of FABP was not achieved in this work. Potential experimentation that can be performed is the completion of further purification. For example, the IEF can be scaled up so as to further separate peaks 1 and 2. Successive chromatography is another option to enhance purification. Also, other chromatography procedures can be performed, such as using a Sephadex G-75 chromatography column. In addition, the FABP can be characterized further. The two IEF peaks can be explored and proven to be FABPs. Ligand-binding and immunoblot assays can also be performed. Further characterization can be carried out by C-13 NMR and HPLC studies. One study would include exploration of the tie in between blood fatty acid content/levels to drug effects on LFABPs by using HPLC studies.

CHAPTER VI

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