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STRUCTURES AND FUNCTION OF FATTY ACID BINDING PROTEINS FROM RAT HEART AND SKELETAL MUSCLE

City University of New York PH.D. 1986

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Structures and Function of Fatty Acid Binding Proteins from Rat Heart and Skeletal Muscle

by

Boctor F. Said

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirement for the degree of Doctor of Philosophy. The City University of New York.

1986

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Abstract

Structures and Function of Fatty Acid Binding Proteins from Rat Heart and Skeletal Muscle

by

Boctor F. Said

Adviser: Professor Horst Schulz

The low molecular weight fatty acid binding proteins (FABP) present in the cytosols of rat heart, liver and skeletal muscle were studied. The three proteins were purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis. Antibodies to the FABP from rat heart and rat liver were used to study the tissue distributions of the heart and liver proteins. This evaluation suggests rat heart-like FABP to be present in heart, skeletal muscle, kidney and stomach, whereas rat liver FABP was identified only in liver and small intestine. Thus, the heart and liver FABPs are immunologically unrelated.

Although FABPs from rat heart and skeletal muscle are immunologically related and have similar amino acid compositions, they are different proteins as judged by their ultraviolet spectra, their electrophoretic mobilities on polyacrylamide gels in the absence or presence of sodium dodecyl sulfate and by their capacities to bind fatty acids before and after delipidation of the proteins. The presence of two moles of Ca^{2+} per mole of skeletal muscle FABP prompted an investigation into the possible identity of this protein with parvalbumin. Based on its Ca^{2+} content pI,

identity of this protein with parvalbumin. Based on its Ca^{2+} content pI, amino acid composition and ultra-violet spectrum, the FABP of rat skeletal muscle is closely related to or identical with rat parvalbumin.

The possible function of rat heart FABP as a cytosolic carrier of fatty acids was assessed by studying the effect of this protein on the transfer of oleic acid from a donor membrane to liposomes. The rate of oleic acid transfer to liposomes was unaffected by the presence of rat heart FABP.

To my dear mother and brothers in Egypt.

Acknowledgement

I wish to express my appreciation to Professor Horst Schulz for his advice, encouragement, and patience. I am also grateful to Dr. Haines, Dr. Russell, Dr. Bittman and Dr. Krakow for their support and assistance. I am also thankful to Dr. Alvin Stern for determining the amino acid composition of fatty acid binding proteins.

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Abbreviations

FABP, fatty acid binding protein; EGTA, ethylene glycol bis(β-amino ethyl ether)-N,N,N',N'-tetra-acetic acid; MOPS, 3-(N-morpholino) propanesulfonic acid; SDS, sodium dodecyl sulfate.

Introduction

Saturated fatty acids are degraded by five sequential reactions. The first reaction is the activation of fatty acids on the outer mitochondrial membrane catalyzed by acyl-CoA synthetase in the presence of CoA and ATP. The acyl residue of acyl-CoA is carried across the inner mitochondrial membrane by carnitine. The activation products are acyl-CoA thioesters. The next step is the dehydrogenation of acyl-CoA by flavine adenine dinucleotide (FAD) which is the cofactor of acyl-CoA dehydrogenase. The reaction product, 2-trans-enoyl-CoA, is hydrated by enoyl-CoA hydratase to yield L-3-hydroxyacyl-CoA. The second dehydrogenation reaction, by which the 3-hydroxyl group is converted to a keto group, is catalyzed by 3-hydroxyacyl-CoA dehydrogenase. The final step is the cleavage of 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase to yield acetyl-CoA and a chain shortened acyl-CoA which undergoes further cycles of B-oxidation until it is completely degraded to acetyl-CoA (Fig. 1).

Free long-chain fatty acids are poorly soluble in water. In the vascular system they are complexed to serum albumin which serves as a physiological carrier for fatty acids throughout the circulatory system (1,2). α-Fetoprotein also exhibits a high binding affinity for long-chain fatty acids, especially for polyunsaturated fatty acids. However, its biological function remains to be elucidated (3,4).

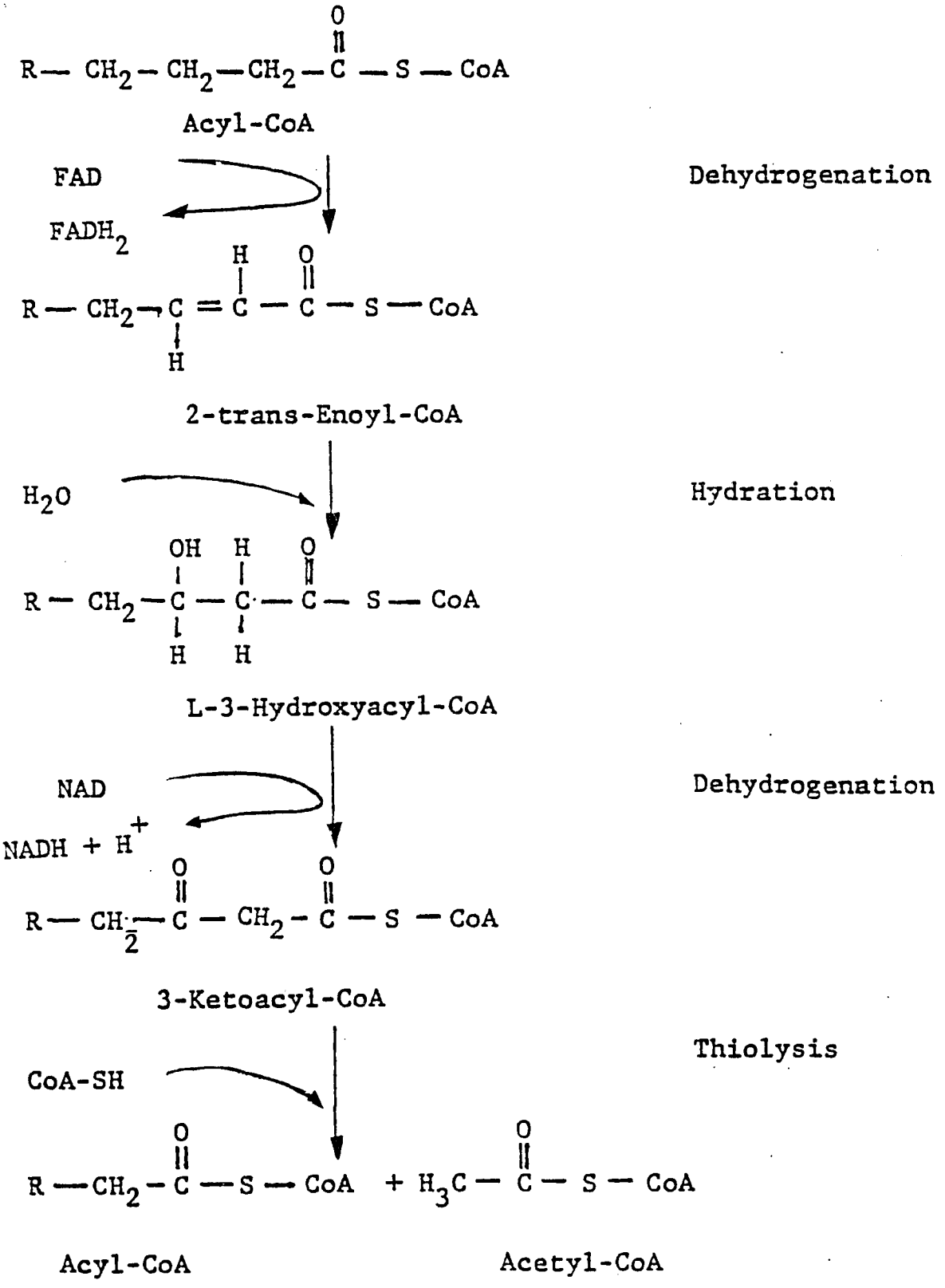


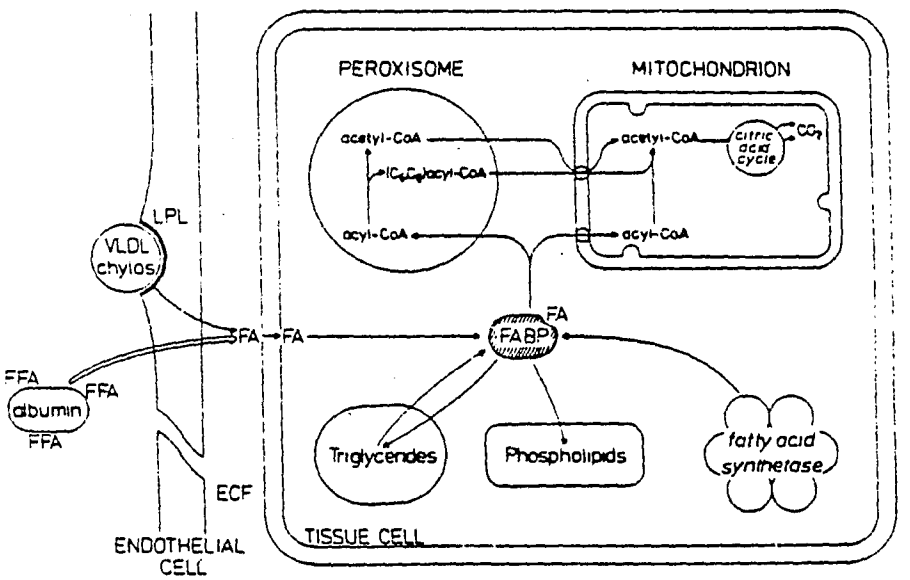
Figure 1 : Pathway of Fatty Acid Oxidation.

The mechanism by which free fatty acids enter cells remains poorly understood. However, once long-chain fatty acids have crossed the plasma membrane, they either diffuse to or are transported to the outer mitochondrial membrane and the endoplasmic reticulum. The existence of a low molecular weight fatty acid binding protein (FABP) in the cytosols of various animal tissues has led to the suggestion (5) that this protein may function as a carrier of fatty acids in the cytosolic compartment as illustrated in Fig. 2.

Fatty acid binding proteins have been identified in the cytosols of rat intestinal mucosa, liver, myocardium, adipose tissue, kidney, mouse preputial glands, chick neural retina, in rat and monkey lung tissues and in rat brain (5-13). The best characterized of these FABPs is that from rat liver which appears to be identical with the anion-binding protein or FABP described by Levi et al. (6) and the amino-azo dye binding protein as described by Ketterer et al. (14). Quantitative radial immunodiffusion analysis and affinity chromatography studies revealed the liver FABP to account for 4-5% of the cytosolic proteins (15,16). Rat liver FABP has been suggested to be identical with the sterol carrier protein (17,18). However, a recent study provided proof for the nonidentity of these two low molecular weight proteins (19). Rat liver FABP is also present in small intestine (20) which additionally, contains the intestinal FABP (21). Recently, I have purified the FABP from rat heart and found it to be different from rat liver FABP (22). Liver FABP shows some homology with the cellular retinoid binding protein and peripheral nerve myelin P protein (23).

Fig. 2. Possible role of FABP (FABP) in the cellular metabolism of long-chain fatty acids.

LDL, very low density lipoprotein; chylos, chylomicrons; LPL,lipoprotein lipase; (F)FA, free fatty acids; ECF, extracellular fluid. The present illustration represented by Glatz et al. (31)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acetyl-CoA
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citric acid cycle
CO_2
IC_2C_nacyl-CoA
acyl-CoA
acyl-CoA
LPL
VLDL chylos
FA
FA
FA
FABP
FFA
FFA
albumin
FFA
Triglycerides
Phospholipids
fatty acid synthetase
ECF
ENDOTHELIAL CELL
TISSUE CELL

Figure 2

Rat liver FABP also binds fatty acyl-CoA thioesters, heme, carcinogen, sulfobromophthalein, bile pigments and several organic anions (24-27). Immunochemical methods were used to detect and quantitate FABPs in rat liver, adipose tissue and jejunum (28-30). All FABPs studied so far have molecular weights close to 14,000 but show large differences in isoelectric points (Table 1 in Ref. 31). Rat liver FABP, for example, exists in three forms, which differ with respect to their isoelectric points (pI 5.2, 7.0, 7.3). All or at least two of these forms may result from the interaction of different ligands or different amounts of ligands with the same protein (7,24,25,32). The binding of long-chain fatty acids to bovine liver FABP (33), human serum albumin (34) and bovine -fetoprotein (35) is also accompanied by changes of the pI to lower values.

The primary structure of rat liver FABP was recently established by analysis of its amino acid sequence (36,37) and the nucleotide sequences of its cDNA (20). The protein consists of a single polypeptide chain composed of 127 amino acid residues with a total molecular weight of 14,184. It has an acetylated NH_2-terminus and contains one free sulfhydryl group.

In general, FABPs have one binding site for long-chain fatty acid. An equilibrium dialysis study by Ketterer et al. (30) showed for rat liver FABP an apparent K_D of 1 uM with palmitate and 0.14 uM with palmitoyl-CoA. Based on the coelution of radioactively-labeled ligands

with cytosolic proteins from a gel filtration column, the K_D values for oleate and palmitoyl-CoA were estimated to be 2.8 uM and 0.24 uM, respectively (7,38). With a binding assay, the apparent K_D for palmitate was determined to be 1.0 uM and 0.8 uM for rat liver FABP and rat heart FABP respectively (39,40). The apparent K_D of pig FABP for 12-doxyl stearate was found to be 0.86 uM by electron spin resonance measurement (41). In bovine liver two distinct FABPs have been identified, one of which has a single binding site for fatty acids whereas the second protein has two sites (33). Because of the poor solubility of fatty acids in aqueous media and their low rate of diffusion through dialysis membranes, a Scatchard plot covering a wide concentration range of fatty acids cannot be obtained by equilibrium dialysis (30).

Definite proof for the physiological role of FABP is still lacking. Several reports have provided indirect evidence for the involvement of FABPs in fatty acid metabolism in liver (42,43). Rat liver FABP and rat intestinal FABP stimulate the microsomal enzymes responsible for synthesis of phospholipids and triacylglycerol. These include mitochondrial and microsomal acyl-CoA synthetase (42), microsomal glycerophosphate acyltransferase (42,44) and diglyceride acyltransferase (45). The influence of liver FABP on these enzymes suggests that FABP may participate in the short term regulation of lipogenesis. It also influences peroxisomal fatty acid oxidation presumably by promoting the transfer of fatty acids into peroxisomes (46).

It is well-known that long-chain fatty acyl-CoA thioesters inhibit various enzyme-catalyzed reactions. Since rat liver FABP binds fatty acyl-CoA thioesters (44) in addition to fatty acids (47), it may also protect enzymes like the adenine nucleotide transporter of mitochondria (48) and enzymes of cholesterol biosynthesis in the endoplasmic reticulum (49) against inhibition by long-chain acyl-CoA thioesters. In cholesterol biosynthesis rat liver FABP reverses inhibitions of hydroxymethylglutaryl-CoA reductase as well as methyl sterol oxidase by long-chain acyl-CoA (49). It also prevents substrate inhibition of acyl-CoA: cholesterol acyltransferase at high concentration of acyl-CoA. Most of the suggestions regarding the physiological role of FABPs have to do with their protection of enzymes against inhibition by long-chain fatty acids and fatty acyl-CoA thioesters.

Feeding of rats with clofibrate and some other hypolipidemic drugs increases the content of hepatic FABP in liver and intestine as well as the rate of fatty acid uptake (50,51). Additionally, clofibrate increases the capacities of rat liver and muscle to oxidize palmitate (52) as well as tissue contents of long-chain acyl-CoA, acetyl-CoA and total CoA in rat liver (53). Cholestyramine feeding also increases the hepatic content of FABP and the rate of triacylglycerol biosynthesis (54).

Following the administration of a high fat-diet, the cytosolic concentration of FABP rises in chick intestine (55) , as well as in liver, small intestine, adipose tissue and rat heart (56,57). The epithelium of rat

small intestine contains two different FABPs, one of which is identical with hepatic FABP whereas the other is a specific intestinal FABP (21,58). The levels of hepatic FABP in liver and intestine are higher in female than in male rats (15,50,59). The intestinal FABP is not affected by sex or clofibrate feeding (40). During diabetes and starvation, hepatic fatty acid oxidation markedly increases and this change is accompanied by a decrease of the level of hepatic FABP and the appearance of a fatty acid binding component with a molecular weight of 400,000 (52,60,61). In both rat liver and heart, the concentration of FABP undergoes diurnal variations with the proteins reaching their highest levels during the dark period (40). The capacity to oxidize palmitate and the activity of citrate synthetase also exhibit similar diurnal periodicities in rat liver and to a lesser extent in rat heart (40).

Fournier et al. (62) have identified a FABP in rat heart with properties similar to that of liver FABP. In contrast, Gloster and Harris (63) have suggested that myoglobin may be the myocardial FABP. Because of my interest in fatty acid metabolism in heart, I set out to purify the FABP from rat liver and heart, to determine their identities and to evaluate the possible function of FABPs in cytosolic transport of fatty acids directed toward oxidation. In addition, I studied the FABP of rat skeletal muscle to evaluate the structural diversity of this type of protein in various tissues of one animal.

Experimental Procedures

Materials

[9,10-^{3}H]palmitic acid (500 mCi/mmol) and [9,10-^{3}H]oleic acid (9.5 Ci/mmol) were purchased from Amersham and New England Nuclear, respectively. Miles laboratory was the source of immunodiffusion plates. Ampholine (pH 4 to 6.5), rabbit parvalbumin, soybean phosphatidylcholine and all standard biochemicals were bought from Sigma. Lipidex-1000 was purchased from Packard Instrument Company. Adult male Sprague Dawley rats (200-250 g) were purchased from Marland Breeding Farms, Hewitt, N .J. Antibodies to rat heart FABP were raised in chicken at the Pocono Rabbit Farm and Laboratory, Canadensis, PA. Rabbit antiserum to rat liver FABP was generously provided by Dr. I. Arias, Liver Research Center, Albert Einstein College of Medicine, Bronx, N.Y. Frozen rat hearts were purchased from Pel-Freez Biologicals. Sephadex G-50 and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Inc.

Purification of Rat Liver FABP

All operations were performed at 4 ^{o}C. Rat liver FABP was purified by a modified version of the procedure published by Trulzsch and Arias (28). Liver from 17 adult male Sprague Dawley rats were cut into small segments and carefully rinsed with 5 mM Tris-HCl (pH 7.4) containing 10 mM mannitol, 70 mM sucrose and 1 mM EGTA. The liver segments were suspended in eight volumes of the same solution and homogenized by three passes of a glass-Teflon homogenizer. The homogenate was centrifuged for 20 min at 27,000 xg and the resulting supernatant was again centrifuged for 90 min at 100,000 xg. The clear solution of cytosolic proteins was concentrated in an Amicon concentrator (PM-10 membrane). To the resulting solution of cytosolic protein (1.4 g) were added 4 nmol of [9,10-^{3}H]oleic acid (9.5 Ci/mmol) dissolved in 4 ul of ethanol. This sample was chromatographed on a Sephadex G-75 column (4 x 42 cm) equilibrated with 10 mM sodium acetate (pH 5.0). Fractions of 5 ml each were collected and assayed for protein and radioactivity. Most of the radioactivity appeared in two peaks. Fractions corresponding to the second peak were pooled and concentrated in an Amicon concentrator (PM-10 membrane). The concentrate was chromatographed on a Sephadex G-50 column (1.5 x 90 cm). Fractions of 4 ml each were collected. Those fractions containing the highest amount of radioactivity were pooled and concentrated in an Amicon concentrator (PM-10 membrane). The resulting concentrate (21.3 mg) was chromatographed on a CM-cellulose column (1.2 x 19 cm) equilibrated with 10 mM sodium

acetate (pH 5). The column was developed with a linear gradient made up of 50 ml of 10 mM sodium acetate (pH 5) and 50 ml of 0.2 M sodium acetate (pH 5). The fractions containing most of the radioactivity were pooled and concentrated in an Amicon concentrator (PM-10 membrane).

Purification of FABP from Rat Heart

All operations were performed at 4 ^{o}C. Purification of the fatty acid binding protein from rat heart was achieved by a modified version of the procedure published by Fournier et al. (62). Hearts from nine adult male Sprague Dawley rats were perfused with Krebs bicarbonate buffer (pH 7.4) for 10 min and rinsed with 5mM Tris-HCl (pH 7.4) containing 210 mM mannitol, 70 mM sucrose, and 1 mM EGTA. The hearts were cut into small sections, suspended in eight volumes of the rinsing solution and homogenized by six passes of a glass-Teflon homogenizer. The homogenate was centrifuged for 20 min at 28,000 xg and the resulting supernatant was centrifuged for 90 min at 110,000 xg. The final preparation of cytosolic proteins was concentrated in an Amicon concentrator (UM-10 membrane). To 30 mg of cytosolic proteins were added 4 nmol of [9,10-^{3}H]oleic acid (817 mCi/mmol) dissolved in 20 ul ethanol. The resulting mixture was passed through a Sephadex G-75 column (2.5 x 42 cm) equilibrated with 5 mM sodium acetate (pH 5). Fractions of 4 ml were collected and those corresponding to the second radioactive peak were pooled. After concentrating these fractions in an Amicon

concentrator (PM-10 membrane) 6 mg of protein were obtained. This material was rechromatographed on a Sephadex G-75 column (1.2 x 47 cm). The fractions containing most of the radioactivity were pooled and concentrated in an Amicon concentrator (UM-2 membrane). The resulting 2.7 mg of protein were applied to a DEAE-cellulose column (0.9 x 7 cm) equilibrated with 10 mM Tris-phosphate (pH 8.3). The column was washed with 200 ml of the same buffer to yield a forerun that contained mostly myoglobin (0.12 mg). The column was then developed with a linear gradient made up of 20 ml each of 10 10 mM Tris-phosphate (pH 6.3) and 20 ml of mM Tris-phosphate (pH 6.3) containing 0.1 M KCl. Since no radioactivity was detected in the eluate, all fractions were combined and concentrated in an Amicon concentrator (UM-2 membrane). The resulting protein sample (1.3 mg) was recharged with [9,10-^{3}H]oleic acid as described above and chromatographed on a CM-cellulose column (0.9 x 7 cm) equilibrated with 10 mM sodium acetate (pH 5). The column was developed with a linear gradient made up of 40 ml of 10 mM sodium acetate (pH 5) and 40 ml of 0.5 M sodium acetate (pH 5). Radioactive material emerged as a symmetrical peak. Fractions containing the radioactive material were pooled and concentrated in an Amicon concentrator (UM-2 membrane) to yield 0.11 mg of pure FABP. In a large scale purification, 200 frozen rat heart were processed at once by the same procedure except that the purification step on DEAE-cellulose was deleted.

Purification of FABP from Rat Skeletal Muscle

All operations were performed at 4 ^{o}C. Skeletal muscle (170 g) from adult rats was trimmed of fat tissue and rinsed with homogenization buffer composed of 5 mM Tris-HCl (pH 7.4), 210 mM mannitol,70 mM sucrose and 1 mM EGTA. The muscle was cut into small pieces and homogenized together with 400 ml of homogenization buffer in a Waring blender for 60 sec at maximum speed. The resulting suspension was again homogenized for 30 sec in batches of 100 ml with a Polytron homogenizer. The resulting homogenate was centrifuged for 20 min at 14,000 xg. The supernatant was saved and the pellet was resuspended in the same buffer, homogenized for 30 sec with a Polytron homogenizer and centrifuged for 20 min at 14,000 xg. The combined supernatants were centrifuged for 90 min at 110,000 xg. The resulting supernatant was concentrated in an Amicon concentrator (YC-05 membrane) to 6 ml which contained 3.8 g of cytosolic proteins. This cytosolic extract was filtered in two batches through a Sephadex G-75 column (4 x 42 cm) which had been equilibrated with 10 mM KPi (pH 7.4). Fractions 45 to 65, which corresponded to the region where low molecular weight proteins would emerge, were combined and concentrated in an Amicon concentrator (YC-05 membrane) to 70 ml which contained 0.95 g protein. The concentrate was rechromatographed in four batches through a Sephadex G-75 column (2.5 x 42 cm) which had been equilibrated with 10 mM KPi (pH 7.4). Fractions of 5.6 ml were collected. Fractions 18 to 30, which corresponded to the region of low molecular weight

proteins, were combined and concentrated in an Amicon concentrator (YC-05 membrane) to 12 ml containing 76 mg protein. After adjusting its pH to 5, this preparation was combined with a solution of 4 nmol of [9,10-^{3}H]oleic acid (500 mCi5/mmol) in 20 ul of ethanol. This partially purified FABP was applied to a CM-cellulose column (1.2 x 15 cm) equilibrated with 10 mM sodium acetate (pH 5). The column was developed with a linear gradient made up of 50 ml of 10 ml sodium acetate (pH 5) and 50 ml of 0.1 M sodium acetate (pH 5). Fractions of 2 ml were collected. FABP to which [9,10-^{3}H]oleic acid was bound emerged from the column in the form of a symmetrical peak (see Fig. 12C). Fractions containing the radioactive material were combined and concentrated in an Amicon concentrator (UM-2 membrane). The yield of pure rat skeletal muscle FABP was 29 mg.

Fractionation of Cytosolic Proteins from Rat Liver and Heart Charged with Either [^{3}H] Palmitic Acid or [^{3}H] Oleic Acid

Cytosolic rat liver or rat heart proteins (2 mg) prepared as described above were dissolved in 1 ml of 10 mM MOPS (pH 7.4) containing 150 mM KCl and mixed with either 2 nmol of [9,10-^{3}H]oleic acid (0.85 uCi/nmol) or 2 nmol of [9,10-^{3}H]palmitic acid (0.5 uCi/nmol). After incubation for 25 min at 37 ^{o}C, the samples were filtered through a Sephadex G-75 column (1.2 x 22 cm) equilibrated with 10 mM MOPS (pH 7.4) containing 150 mM KCl. Fractions of 1 ml were collected and assayed for protein and radioactivity. After each experiment bovine

serum albumin (1 mg/ml) was passed through the column to remove traces of radioactive fatty acids bound to the column material.

Determination of Fatty Acid Binding to Purified and Partially Purified FABPs from Heart and Skeletal Muscle

Partially purified FABP (3 mg) after two filtrations through Sephadex G-75 or pure FABP (1 mg) dissolved in 1 ml of 10 mM KPi (pH 7.4) was combined with 2.1 nmol of either [9,10-^{3}H]oleic acid (0.5 Ci/mmol) or [9,10-^{3}H]palmitic acid (0.5 Ci/mmol) dissolved in 10 ul of ethanol. After incubation for 1 hr at 37 ^{o}C, the sample was passed through a Sephadex G-75 column (1.2 x 22 cm) equilibrated with 10 mM KPi (pH 7.4). Fractions of 1 ml were collected and assayed for protein as well as radioactivity. After each experiment a solution of bovine serum albumin (1 mg/ml) was passed through the column to remove traces of radioactive fatty acids bound to the column material.

Delipidation of Pure FABPs

Purified FABPs (4 mg) from rat heart or skeletal muscle were delipidated by passing them through a Lipidex-1000 column (0.6 x 13 cm) equilibrated with 10 mM KPi (pH 7.4). Free and protein-bound fatty acids are retained on the column when this procedure is performed at 37 ^{o}C (39).

Preparation of Cytosolic Extracts from Various Rat Tissues

Heart, kidney, spleen and liver from rat were rinsed with a solution containing 5 mM Tris-HCl (pH 7.4), 210 mM mannitol, 70 mM sucrose and 1 mM EGTA. After cutting the tissues into small pieces with a pair of scissors, they were suspended in eight volumes of rinsing solution and homogenized by six passes of a glass-Teflon homogenizer. Stomach and small intestine from rat were inverted, cleaned, washed with the rinsing solution and homogenized with a Polytron homogenizer for 40 sec in eight volumes of rinsing solution. Red skeletal muscle and adipose tissue from rat were washed with rinsing solution and homogenized for 40 sec together with eight volumes of rinsing solution with a Polytron homogenizer. All tissue homogenates were centrifuged at 14,000 xg for 20 min. The resulting supernatants were then centrifuged at 110,000 xg for 90 min and concentrated in an Amicon concentrator (YC-05 membrane).

Ouchterlony Immunodiffusion

Chicken antiserum (1.32 mg) to rat heart FABP or rabbit antiserum (0.85 mg) to rat liver FABP were placed in the center wells of Ouchterlony immunodiffusion plates which were covered with a wet sponge to prevent the agar from drying out. The antibodies were allowed to diffuse into the agar for 5 hrs at 5 ^{o}C. The antigens were placed in the outer wells and the plates were kept for 2 to 3 days at 5 ^{o}C until the immunoprecipitates were visible. The plates were washed with 1 mM

KPi (pH 7.2) containing 0.9 % NaCl for 48 hrs at 5 ^{o}C to remove soluble proteins. After drying the plates, they were stained for 15 min with a 0.5% solution of Amido Black and then destained with a 1:1 mixture of water and methanol which contained 1% acetic acid.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed on 6.2% polyacrylamide gels at pH 8.6 as described in principle by Davis (64). Gels were stained for 30 min with 2% Coomassie brilliant blue in 7% acetic acid and destained with 7% acetic acid. Electrophoresis on 16.3% polyacrylamide gels in the presence of SDS was carried out as described by Laemmli (64). Isoelectric focusing was performed with 7.5% polyacrylamide gels, which contained Ampholine (pH 4-6.5), as described in principle by Wrigley (66). Glutamic acid (10 mM) and histidine (10 mM) served as anode and cathode buffer, respectively. After electrophoresis of the gel for 2.5 hrs at 400 V, a 1 cm wide strip was cut vertically from the slab gel. The gel strip was divided into 1 cm wide segments each of which was added to 1 ml of deionized water. After incubating the gel segments for 2 hrs, the pH values of the resulting solutions were determined. The main portion of the slab gel was washed twice for 30 min each at 30 C^{o} with a mixture of ethanol, water and acetic acid in the ratio of 35:55:10 to remove the Ampholine. The gel was then treated with 10% trichloroacetic acid solution for 1 hr, stained for 30 min with 0.6% Coomassie brilliant blue in 7% acetic acid, and finally destained in 7% acetic acid.

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (67).

Calcium Determination

Rat skeletal muscle FABP before and after delipidation with Lipidex-1000 was dialyzed for 24 hrs against 3 mM sodium acetate (pH 7.4). The Ca^{2+} content of the FABP solution and the dialysis buffer were determined by atomic absorption spectrophotometry at Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

Amino Acid Compositions

The amino acid composition of the FABPs from rat heart and skeletal muscle were determined by Dr. A. Stern, Roche Institute of Molecular Biology, Nutley, N.J. Amino acids were separated by high performance liquid chromatography and quantitated with fluorescamine (68).

Preparation of Liposomes

Liposomes were prepared by a modified procedure of Barrow and Lentz (69). Soybean phospholipids (40 mg) were dissolved in 1 ml chloroform. The solvent was then removed under a nitrogen stream so that the lipids formed a thin layer on the inner surface of a test tube.

Four ml of 10 mM MOPS buffer (pH 7.4) containing 0.1 M KCl were added to the test tube and vigorously mixed for 40 min after which time the suspension was sonicated to clarity in a bath-type sonicator (Laboratory Supply Co., Hicksville, N.Y.) at 10-15 ^{o}C.

Transfer of Oleic Acid from a Fixed Donor Membrane to Liposomes

The fixed donor membrane was prepared by immersing a cylindrical sheet of polypropylene (2 x 0.5 cm) in a solution of soybean phospholipids (25 mg/ml) and 4 uM [9,10-^{3}H]oleic acid (0.5 Ci/mmol) in chloroform for 6 min. The solvent was then removed under a stream of nitrogen. The resulting donor membrane was incubated at 37 ^{o}C in 4 ml of a solution containing 10 mM MOPS (pH 7.4) , 0.1 M KCl and liposomes (5 mg/ml) in the presence or absence of partially purified rat heart FABP (1 mg/ml). At various times, 0.25 ml of the incubation mixture containing liposomes and FABP was removed and applied to a Sephacryl-S-300 column (20 x 0.6 cm) equilibrated and developed with 10 mM KPi buffer (pH 7.4). Fractions of 0.65 ml each were collected and assayed for radioactivity and protein concentration. Liposomes emerged in the void volume, whereas FABP was resolved from liposomes and eluted in later fractions.

Results

Identification of Low-Molecular-Weight Fatty Acid Binding Proteins (FABPs) in the Cytosols of Rat Liver and Heart

When the cytosolic proteins from rat liver were charged with [^{3}H]oleic acid and separated by chromatography on Sephadex G-75, the radioactive material emerged from the column in two peaks (see Fig 3A). The first peak according to its position corresponds to serum albumin, whereas the second peak was eluted where FABP with a molecular weight of 14,272 would be expected to emerge. An experiment in which the cytosolic rat liver proteins were charged with [^{3}H]palmitic acid instead of [^{3}H]oleic acid gave similar results (see Fig. 3B), especially when the higher specific radioactivity of the latter fatty acid is considered. When the cytosolic fraction of rat heart was charged with [^{3}H]oleic acid and analyzed by fractionation on Sephadex G-75, a similar picture was observed (see Fig. 3B). However, compared to liver, more radioactivity was associated with the first peak, especially when the hearts were homogenized without being first perfused. This observation together with the fact that the position of the first peak corresponds to that of serum albumin leads to the conclusion that the first radioactive peak to emerge from the column reflects the binding of fatty acids to residual serum albumin. Finally, I tested bovine heart myoglobin for its ability to bind oleic acid.

Fig. 3. Gel filtration on Sephadex G-75.

A, Cytosolic rat liver proteins; B, cytosolic rat heart proteins; C, bovine heart myoglobin. For experimental details see "Experimental Procedures". Protein concentration (●); [9,10-^{3}H]oleic acid (▲) and [9,10-^{3}H]palmitic acid (■)

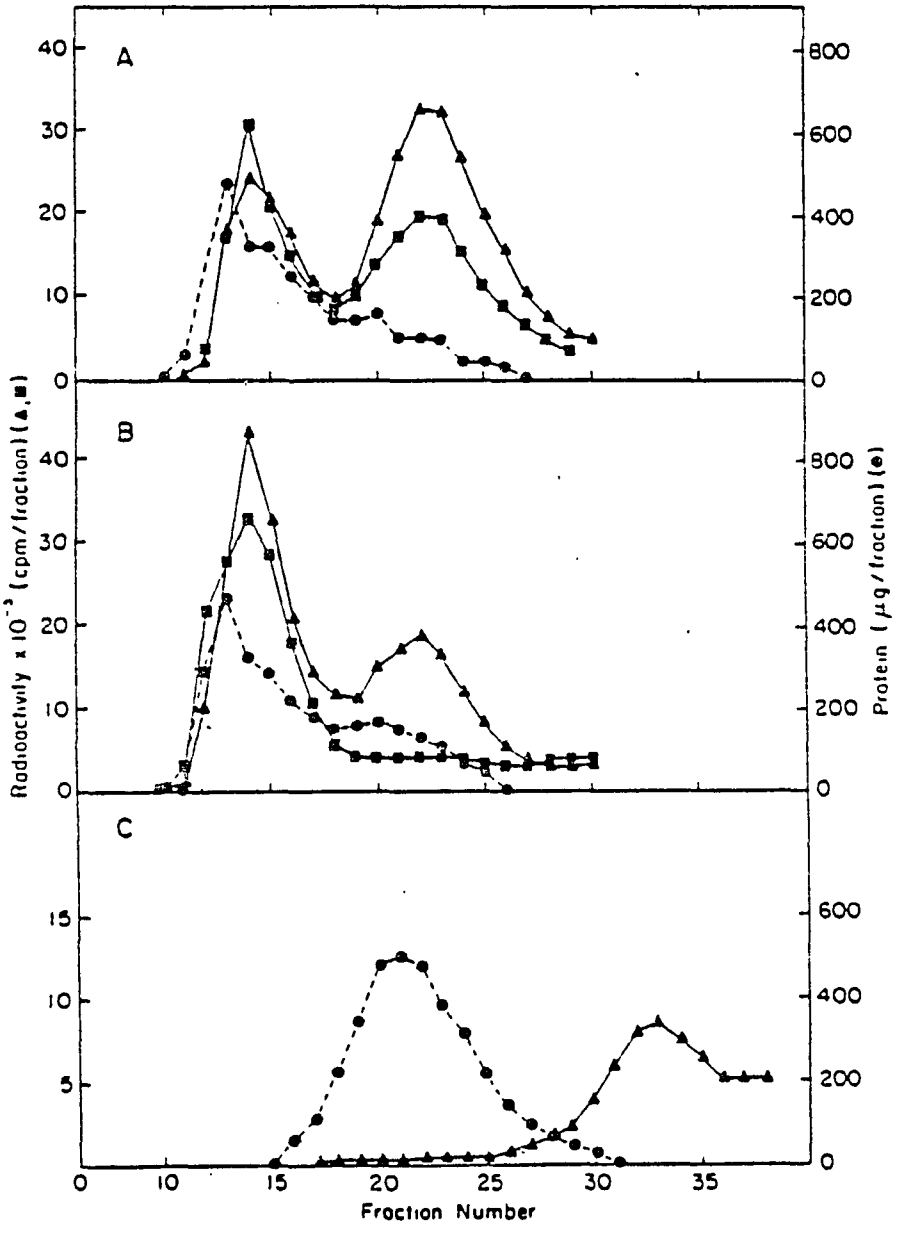
A
B
C
Radioactivity x 10^{-3} (cpm/fraction) (▲,■)
Protein (μg/fraction) (●)
Fraction Number

Figure 3

As demonstrated in Fig 3C bovine heart myoglobin has no measurable affinity for this fatty acid. This result raises doubts about the reported binding of fatty acids to myoglobin (63).

Purification and Comparison of the Low-Moleclar-Weight Fatty Acid Binding Proteins from Rat Heart and Liver.

Fatty acid binding protein from rat liver was purified to near homogeneity by the procedure of Trulzsch and Arias (28) with some modifications. In the last purification step on CM-cellulose all radioactivity was co-eluted with the major protein fraction, presumably FABP, which was well resolved from a minor protein contaminant (see Fig. 4). When subjected to polyacrylamide gel electrophoresis in the presence of SDS this preparation of FABP gave rise to one major band which accounted for more than 90% of the applied protein (see Fig. 5, lane 1). On polyacrylamide gel electrophoresis under non-denaturing conditions rat liver FABP was resolved into two major and one minor fractions (see Fig. 6, lane 1). This result is in good agreement with the reported separation of purified rat liver FABP into three or four immunologically identical fractions on isoelectric focusing (28).

The purification of the FABP from rat heart was achieved by a modified version of the procedure of Fournier et al. (62). Passing the cytosolic heart proteins twice through a Sephadex G-75 column yielded a fraction of low-molecular-weight proteins which had the capacity to bind oleic acid and palmitic acid (see Fig. 7A and 8A).

Fig. 4. Chromatography of rat liver FABP on CM-cellulose.

Protein concentration (●) and [9,10-^{3}H]oleic acid (▲). For experimental details see "Experimental Procedures".

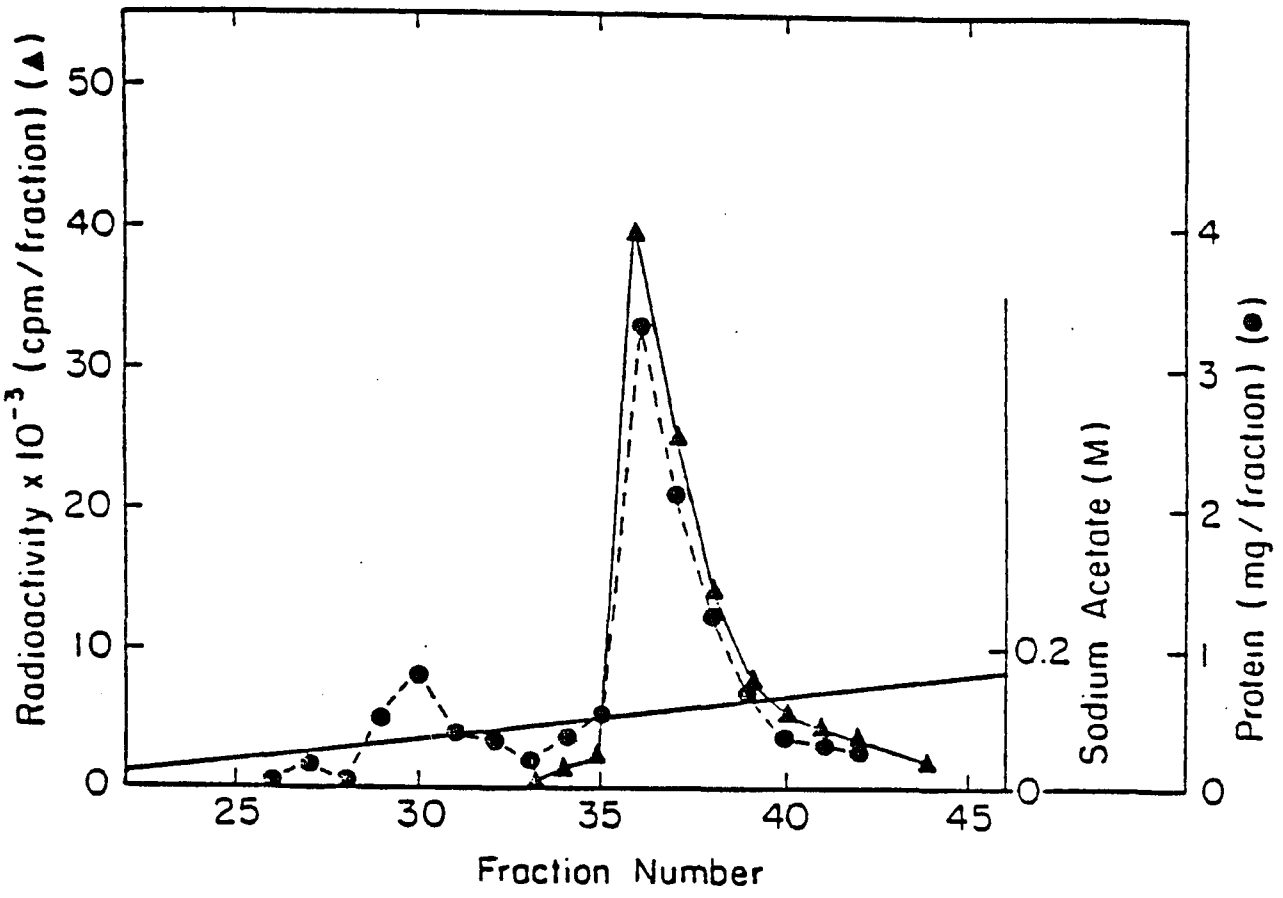
Radioactivity x 10^{-3} (cpm / fraction) (▲)
Sodium Acetate (M)
Protein (mg / fraction) (●)
Fraction Number
0
10
20
30
40
50
0.2
1
2
3
4
25
30
35
40
45

Figure 4

Fig. 5. Polyacrylamide disc gel electrophoresis in the presence of SDS.

Lane 1, liver FABP (15 μg); lane 2, liver and heart FABP; lane 3, liver and skeletal muscle FABP; lane 4, skeletal muscle FABP (15 μg); lane 5, heart and skeletal muscle FABP; lane 6, heart FABP (12 μg).

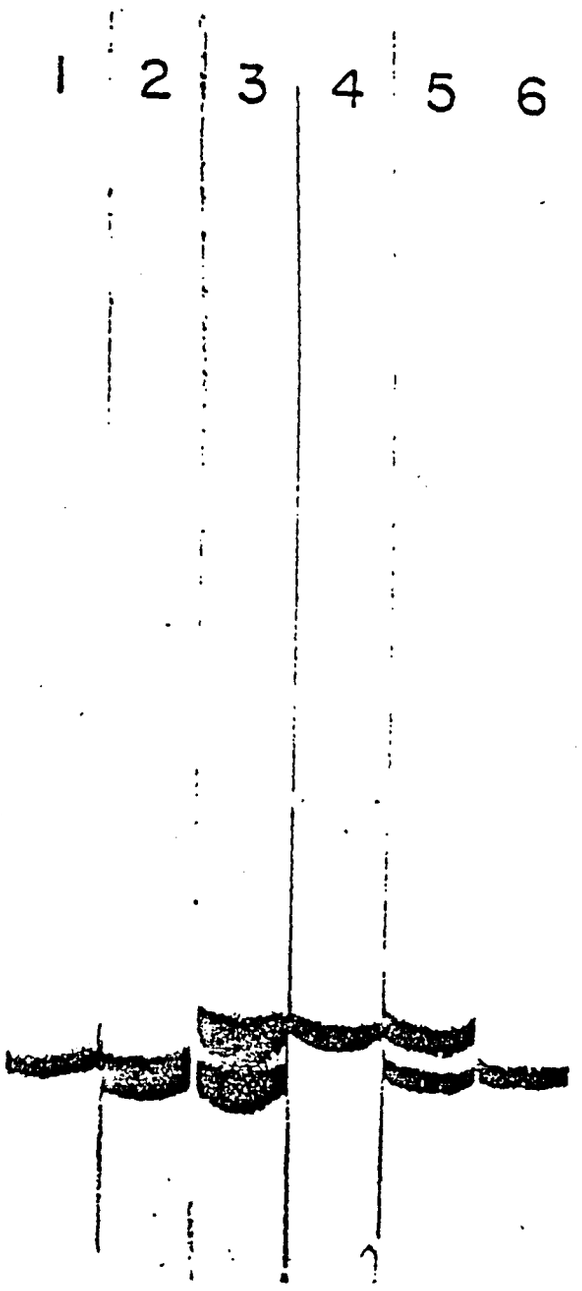

Figure 5

Fig. 6. Polyacrylamide disc gel electrophoresis under non-denaturing conditions.

Lane 1, purified FABP from rat liver (18 μg); lane 2, purified FABP from rat heart (12 μg); lane 3, partially purified FABP from rat heart after the second filtration through Sephadex G-75 (48 μg); lane 4, rat heart myoglobin (15 μg). For experimental details see "Experimental Procedures".

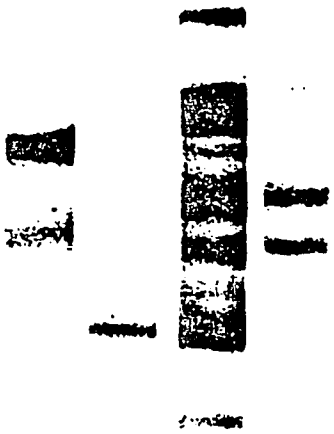

Figure 6

The absorbance of this fraction was indicative of the presence of hemoproteins, most likely myoglobin. On polyacrylamide gel electrophoresis under non-denaturing conditions this protein was separated into more than seven proteins (see Fig. 6, lane 3). This partially purified preparation of FABP was further purified by chromatography on DEAE-cellulose, on which myoglobin was removed, and finally on CM-cellulose from which the FABP emerged as a symmetrical peak. In large scale purification (200 rat hearts), chromatography of the partially purified FABP on CM-cellulose column yielded a protein fraction which emerged as a symmetrical peak (see Fig. 8C). The final preparation of heart FABP behaved as a homogeneous protein on polyacrylamide gel electrophoresis in the absence (Fig. 6, lane 2) and in the presence of SDS (Fig. 5 lane 6). On polyacrylamide gel electrophoresis in the absence of SDS, the myoglobin fraction gave rise to three bands (see Fig. 6, lane 4) all of which were visible before staining for protein. Both myoglobin and purified FABP from rat heart were recharged with [^{3}H]oleic acid and rechromatographed on a small Sephadex G-75 column. Since little protein was available, only the radioactivity eluted from the column was measured. As can be seen in Fig. 7B, curve 1, the first peak of radioactivity was centered around fraction 20 where the FABP is expected to emerge (compare Fig. 7A and B). The second radioactive peak was most likely due to unbound fatty acids which were always observed to emerge in later fractions. A similar experiment with rat heart myoglobin proved that no radioactivity was associated with this protein (see Fig. 7, curve 2) which was eluted in fractions 15-25 (for comparison, see Fig. 3C). All radioactivity which emerged in later fractions is characteristic of unbound fatty acids.

Fig. 7. Gel filtration of rat heart FABP and myoglobin on Sephadex G-75.

A, Partially purified rat heart FABP corresponding to the second radioactive peak shown in Fig. 3B. B1, Purified rat heart FABP. B2, Rat heart myoglobin. For experimental details see "Experimental Procedures".

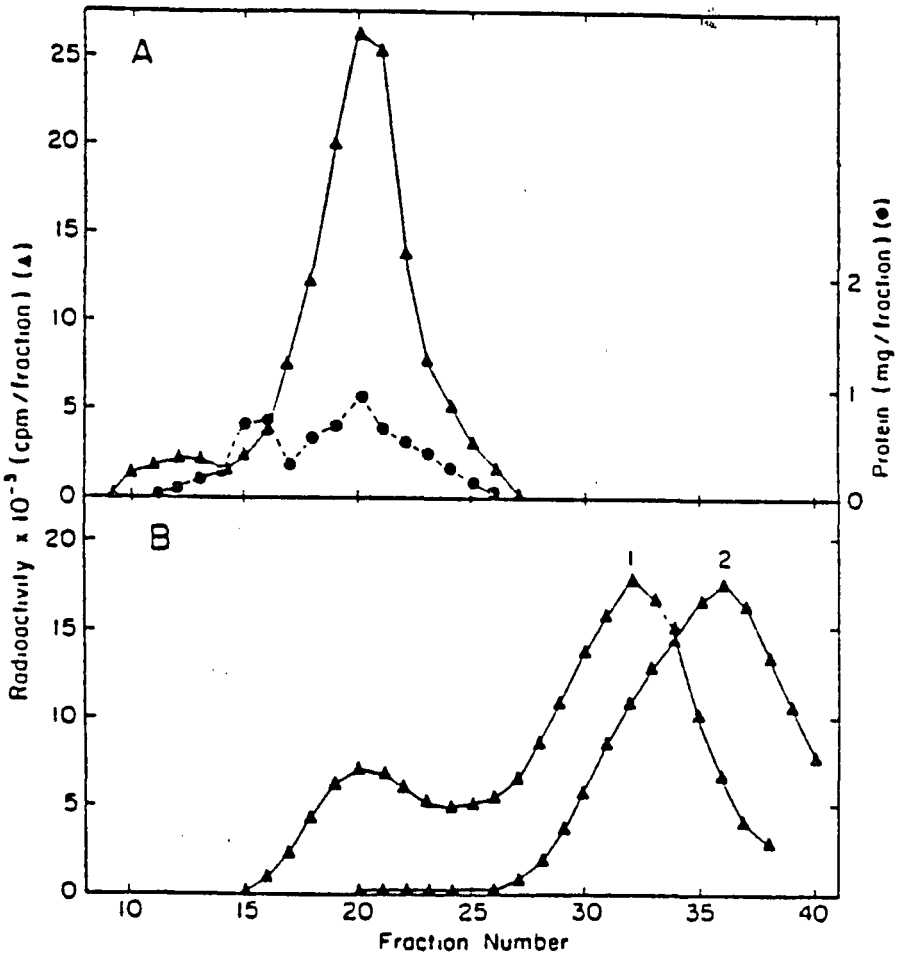
A
B
1
2
Radioactivity x 10^{-3} (cpm/fraction) (▲)
Protein (mg/fraction) (●)
Fraction Number

Figure 7

Fig. 8. Purification of FABP from rat heart.

A. Chromatography of partially purified FABP on Sephadex G-75.

B. Chromatography of pure FABP on Sephadex G-75.

C. Chromatography of partially purified FABP on CM-cellulose.

(-x-) Protein; (●) [9,10-^{3}H]oleic acid; (▲) [9,10-^{3}H]palmitic acid.

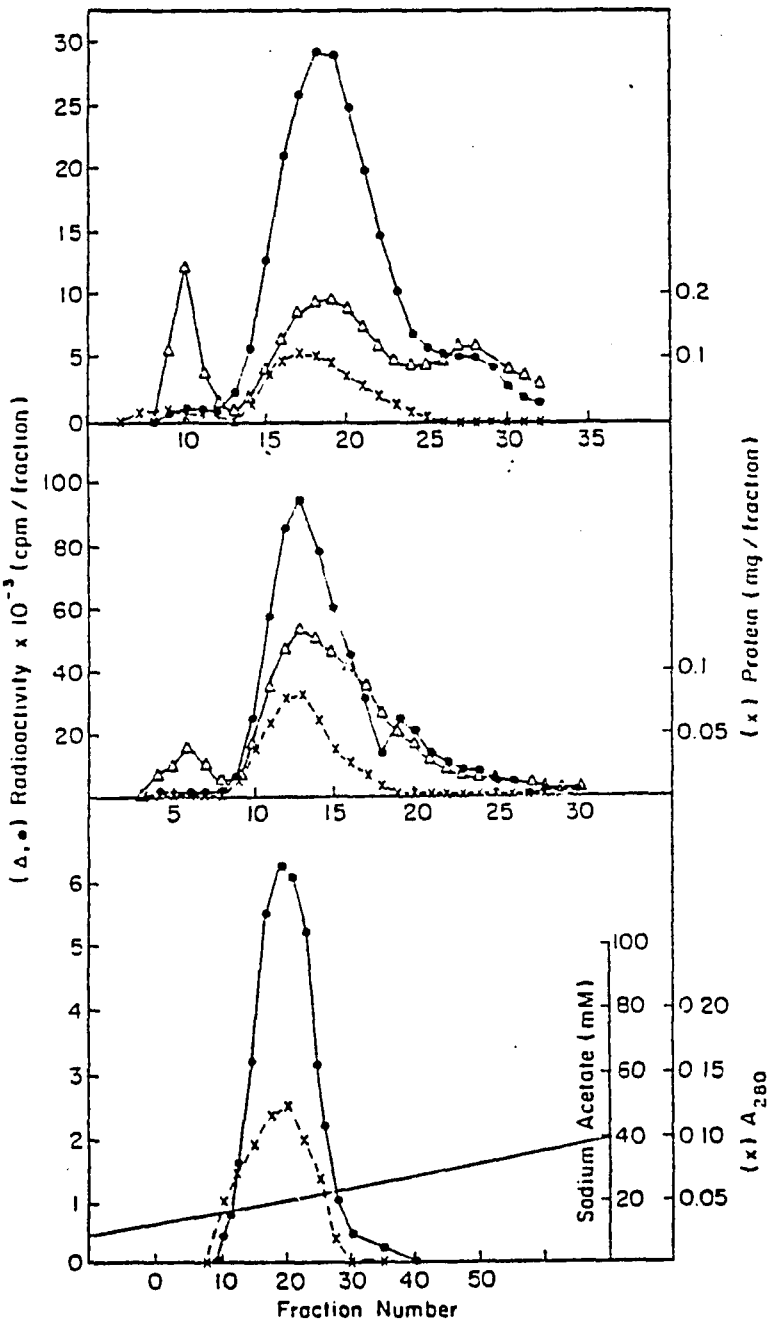
(Δ,●) Radioactivity x 10⁻³ (cpm / fraction)
(x) Protein (mg / fraction)
Sodium Acetate (mM)
(x) A280
Fraction Number

Figure 8

The structural relationship between rat liver FABP and rat heart FABP was also evaluated by Ouchterlony double immunodiffuson experiments (71). The picture of an Ouchterlony plate shown in Fig. 9 demonstrates the formation of strong precipitin lines between antibodies to rat liver FABP (center well) and purified rat liver FABP (well 2) as well as cytosolic rat liver proteins (well 1 and 3). In contrast, neither purified rat heart FABP (well 4 and 5) nor cytosolic rat heart proteins gave rise to a corresponding precipitin line. A very weak precipitin line close to the antibody well was seen with cytosolic rat heart proteins. This line is not visible in Fig. 9. Since this precipitin line was not observed with pure heart FABP, it must be due to an antigen other than the FABP.

Tissue Distribution of Rat Liver and Rat Heart FABPs.

The presence of rat liver and rat heart FABPs in the cytosols of various rat tissues was studied by Ouchterlony immunodiffusion. Chicken antibodies to rat heart FABP and rabbit antibodies to rat liver FABP were used in this survey of rat tissues which included liver, heart, skeletal muscle, kidney, stomach, small intestine, spleen, and adipose tissue. Antibodies to rat liver FABP formed immuno-precipitates with the cytosolic extracts of liver and small intestine (see Fig.10), both of which have been shown to contain rat liver.

Fig. 9. Ouchterlony immunodiffusion analysis of the low-molecular weight FABPs from rat heart and liver.

The center well contained antibodies (0.84 mg) to rat liver FABP raised in rabbit. Antigen: Well 1, cytosolic rat liver proteins (40 μg); well 2, purified rat liver FABP (4 ug); well 3, cytosolic rat liver proteins (10 μg); well 4 and 5, purified rat heart FABP (1.1 μg and 2.2 ug, respectively); well 6, cytosolic rat heart proteins (40 μg).

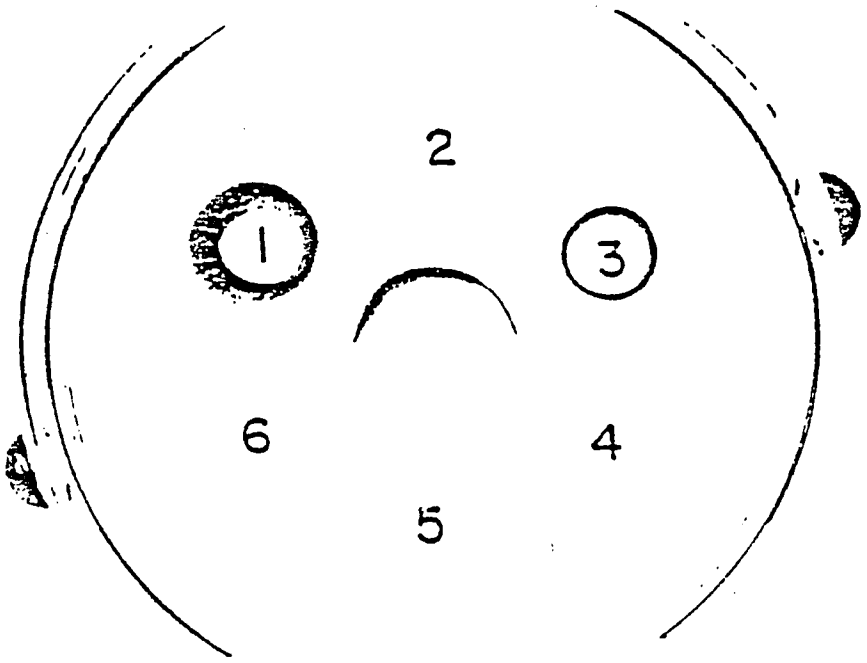

Figure 9

Fig. 10. Ouchterlony immunodiffusion analysis.

The center well contained rabbit antiserum (0.85 mg) to rat liver FABP. The outer wells contained cytosolic protein of (L) liver (160 μg), (I) small intestine (134 μg), (S) stomach (104 μg), (K) kidney (174 μg), (M) skeletal muscle (136 μg), and (A) adipose tissue (90 μg).

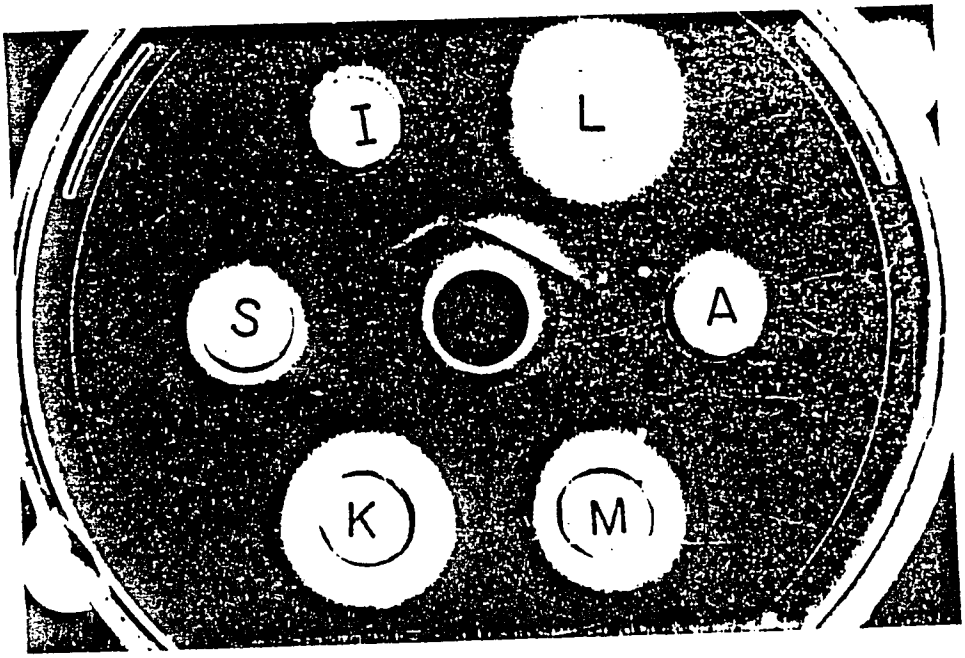

Figure 10

Fig. 11. Ouchterlony immunodiffiusion analysis.

The center well contained chicken antiserum (1.32 mg) to rat heart FABP. The outer wells contained cytosolic proteins of (H) heart (12 μg), (K) kidney (172 μg), (L) liver (120 μg), (I) small intestine (30 μg), (S) stomach (130 μg) and (M) skeletal muscle (204 μg).

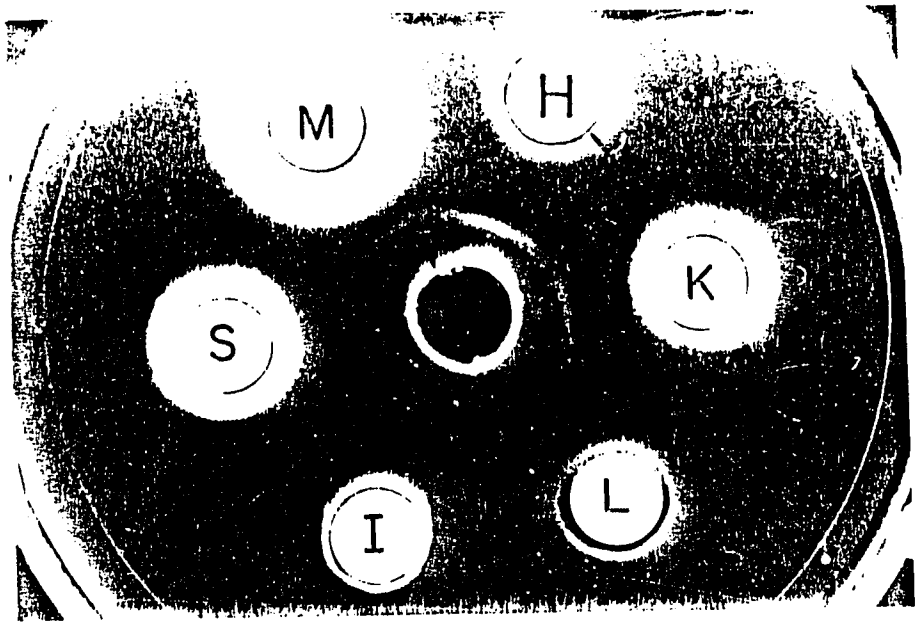

Figure 11

All other tissues tested do not seem to contain rat liver FABP at levels detectible by our experimental approach. Even with a cytosolic extract of adipose tissue, which has been reported to contain a liver-type FABP (11), no precipitin line was observed (data not shown). Chicken antibodies to rat heart FABP formed an immunoprecipitate with a cytosolic extract of heart muscle and in addition cross-reacted with antigens present in the cytosols of skeletal muscle, kidney, and stomach (see Fig. 11). No antigens cross-reacting with antibodies to heart FABP were detected in the cytosolic fractions of liver, small intestine, adipose tissue, and spleen.

Purification and Characterization of FABP from Rat Skeletal Muscle

An extract of soluble cytosolic proteins was prepared from rat skeletal muscle and filtered twice through a Sephadex G-75 column to partially purify the low molecular weight fraction which consisted mainly of myoglobin and FABP. This fraction binds fatty acids as demonstrated by the co-elution of protein and either radioactive palmitic acid or oleic acid (see second peak of radioactivity in Fig. 12A). The first and third peaks of radioactivity seem to be due to the elution of aggregate and monodisperse free fatty acids, respectively. Chromatography of the partially purified FABP on CM-cellulose yielded a protein fraction which emerged from the column in the form of a symmetrical peak coincident with the peak corresponding to radioactive oleic acid (see Fig. 12C). Myoglobin remained bound to the column material. The protein thus purified emerged from a Sephadex G-75 column at a position characteristic of FABP and had the capacity to bind oleic acid as well as palmitic acid (see Fig. 12B).

Fig. 12. Purification of FABP from rat skeletal muscle.

A. Chromatography of partially purified FABP on Sephadex G-75.

B. Chromatography of pure FABP on Sephadex G-75.

C. Chromatography of partially purified FABP on CM-cellulose.

For experimental details see "Experimental Procedures". (0) Protein; (▲) [9,10-^{3}H]oleic acid; (▬)[9,10-^{3}H] palmitic acid.

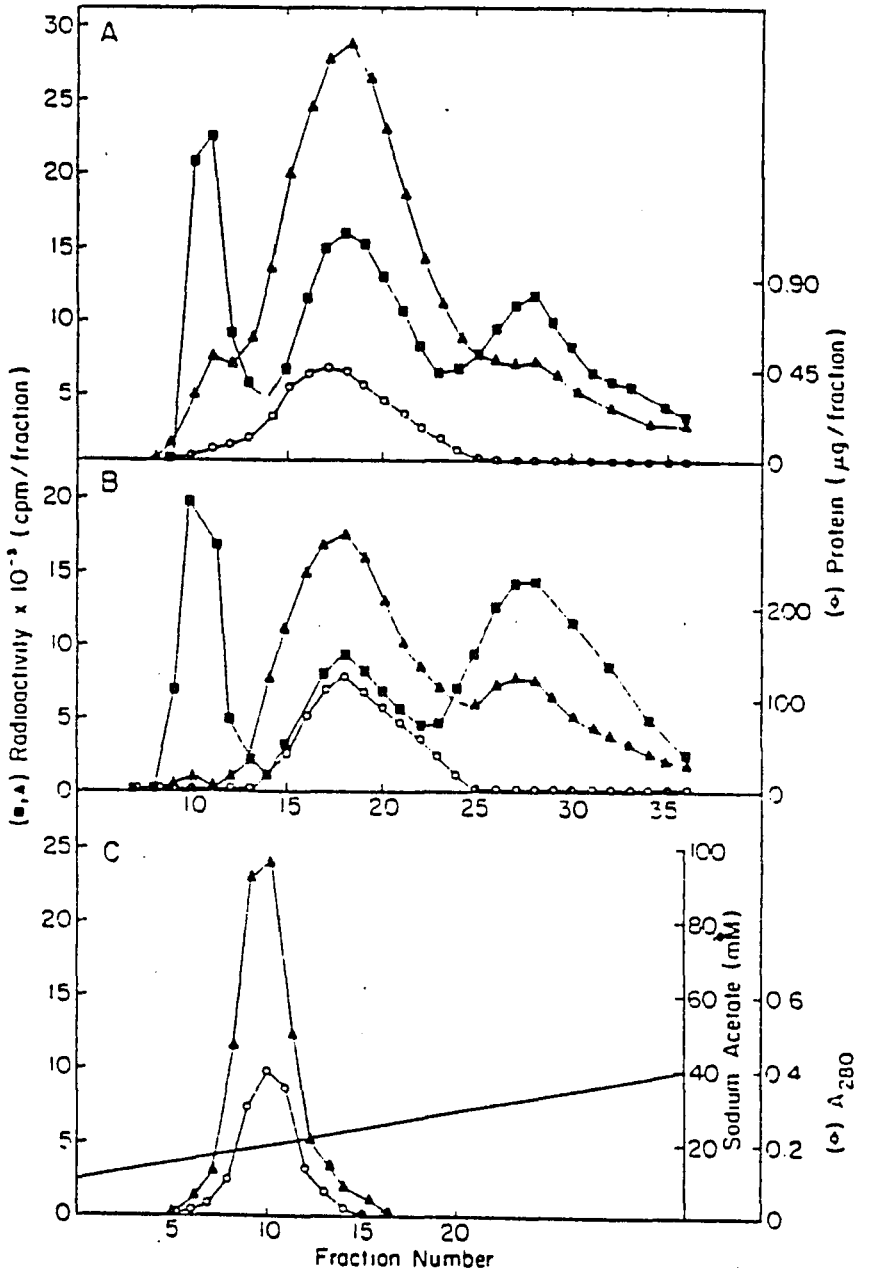
A
B
C
(■,▲) Radioactivity x 10⁻³ (cpm/fraction)
(○) Protein (μg/fraction)
Sodium Acetate (mM)
(○) A280
Fraction Number

Figure 12

Since the purified skeletal muscle protein cross-reacted with antibodies to rat heart FABP (see Fig. 13), it is the heart type FABP originally identified immunologically in a cytosolic extract of rat skeletal muscle (see Fig. 11). It is noteworthy that the immunoprecipitation line due to skeletal muscle FABP is weaker and formed at a different position than the precipitin line formed with heart FABP (see Fig. 13) even though identical amounts of purified FABPs were used. This observation suggests that either an impurity present in the preparation of skeletal muscle FABP reacted with the antibodies or that these two FABPs are not identical proteins.

Polyacrylamide gel electrophoresis under non-denaturing conditions proved the skeletal muscle FABP to be a homogeneous protein (see Fig. 14, lane 2). However, the skeletal muscle FABP migrated differently than the heart fatty acid binding protein (compare lane 1 and 2 in Fig. 14) which previously was shown to be a different protein than liver FABP (22). Poly- acrylamide gel electrophoresis in the presence of SDS provided further proof for the nonidentity of the FABPs from rat heart and skeletal muscle (see Fig. 5). Although the heart and liver FABPs co-migrated (see Fig. 5, lane 2), the skeletal muscle FABP was clearly separated from the heart and liver FABPs (see Fig. 5, lane 3 and 5).

Fig. 13. Ouchterlony immunodiffusion analysis.

The center well contained chicken antiserum (1.32 mg) to rat heart FABP. The outer wells contained (1) pure rat skeletal muscle FABP (5 μg), (2) pure rat heart FABP (5 μg), (3) cytosolic proteins of rat skeletal muscle (204 μg), (4) cytosolic proteins of rat heart (112 μg).

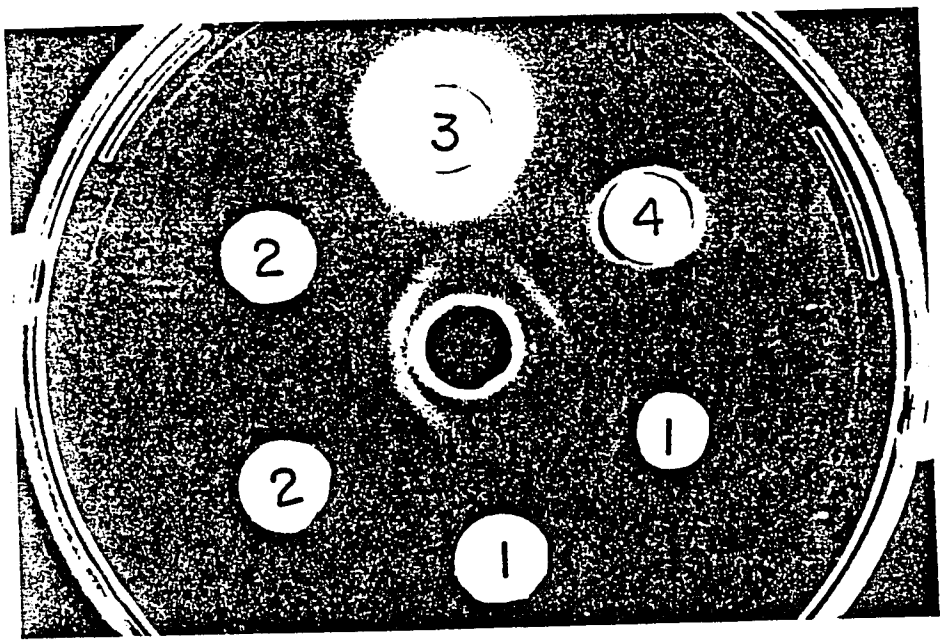

Figure 13

Fig. 14. Polyacrylamide disc gel electrophoresis of purified rat FABPs under non-denaturing conditions.

Lane 1, heart FABP (12 μg); lane 2, skeletal muscle FABP (15 μg); lane 3, liver FABP (15 μg); lane 4, mixture of liver FABP (15 μg), heart FABP (12 μg), and skeletal muscle FABP (15 μg).

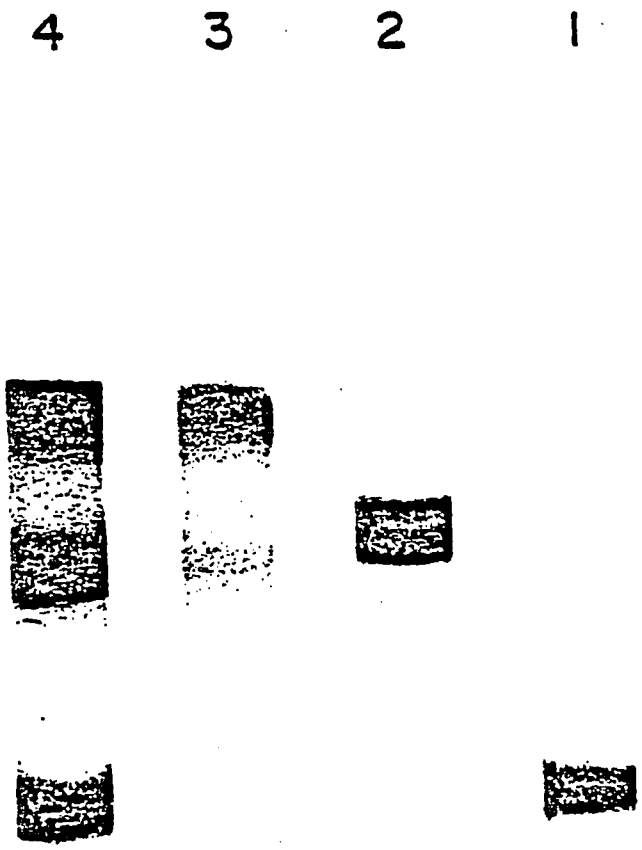

Figure 14

The FABPs from rat heart and skeletal muscle were also compared for their capacities to bind fatty acids. The proteins were first delipidated by passing them through a Lipidex-1000 column at 37 ^{o}C and thereafter were charged with less than stoichiometric amounts of either oleic acid or palmitic acid. As can be seen in Fig. 15A, heart FABP bound all oleic acid and most of the palmitic acid. The small peak of palmitic acid preceding the main peak seems to be due to an aggregated form of unbound fatty acid. Under virtually identical conditions only a small fraction of either oleic acid or palmitic acid was bound by skeletal muscle FABP. The larger part of palmitic acid emerged from the column as free fatty acids behind the FABP (see Fig. 15B). Although these experiments do not provide quantitative binding data, they clearly demonstrate that the FABP from rat skeletal muscle as compared to rat heart FABP has a much lower affinity for saturated as well as unsaturated fatty acids. This result provides additional evidence for the nonidentity of the FABPs from rat heart and skeletal muscle. This conclusion is also supported by the ultraviolet spectra of the purified FABPs from rat heart, liver and skeletal muscle shown in Fig. 16.

Fig. 15. Binding of fatty acids to delipidated FABPs from heart and skeletal muscle

A, Purified rat heart FABP (1.1 mg). B, Purified FABP from rat skeletal muscle (1.4 mg). The FABPs were charged with (C18:1) 2.1 nmol [9,10-^{3}H]oleic acid (0.5 μCi/nmol) or (16:0) 2.1 nmol [9,10-^{3}H]palmitic acid (0.5 μCi/nmol) and passed through a Sephadex G-75 column (1.2 X 22 cm) as described under Experimental Procedures.

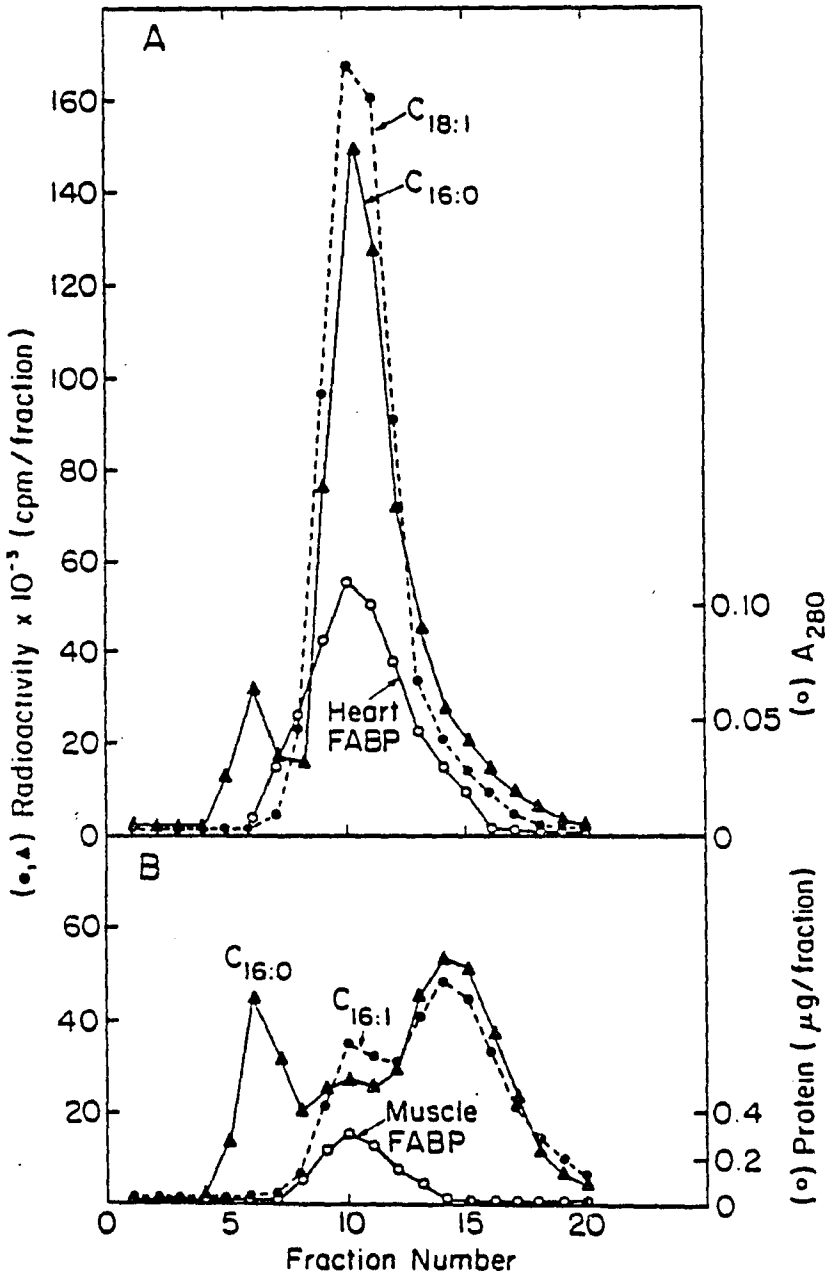
A
B
$C_{18:1}$
$C_{16:0}$
Heart FABP
Muscle FABP
(•,▲) Radioactivity x 10^{-3} (cpm/fraction)
(○) A_{280}
(○) Protein (μg/fraction)
Fraction Number
0
20
40
60
80
100
120
140
160
0.05
0.10
0.2
0.4
5
10
15
20

Figure 15

Fig. 16. Ultraviolet spectra of rat FABPs from heart, liver and skeletal muscle.

Solutions contained 0.5 mg of FABP in 1 ml of 10 mM sodium acetate (pH 5).

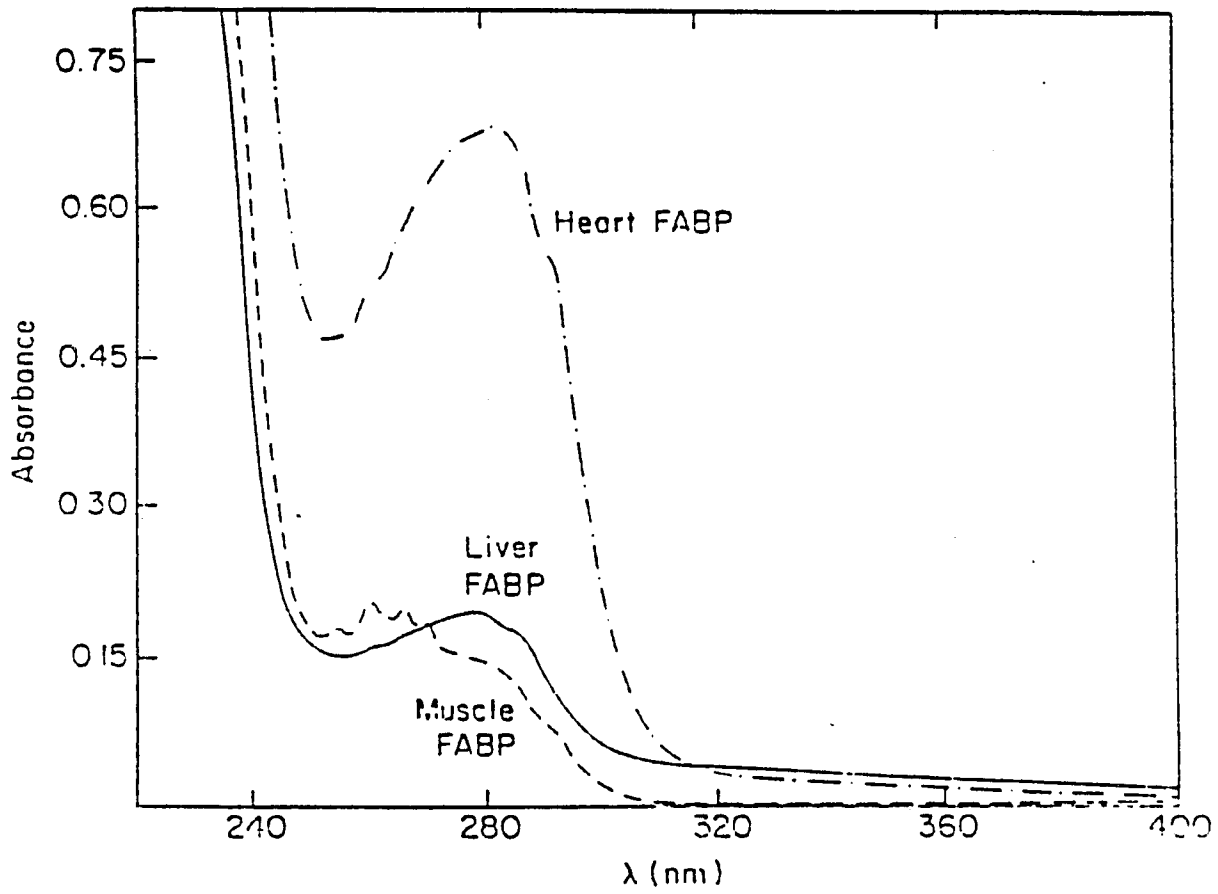
0.75
0.60
0.45
0 30
0 15
Absorbance
Heart FABP
Liver
FABP
Muscle
FABP
240
280
320
360
400
λ (nm)

Figure 16

Is the FABP from Rat Skeletal Muscle Identical with Parvalbumin ?

The ultraviolet spectrum of skeletal muscle FABP is characteristic of a protein which contains several phenylalanine and tyrosine residues, but is devoid or nearly devoid of tryptophan. This observation together with the fact that the FABP is a major low molecular weight protein of skeletal muscle prompted me to study the possible identity of this protein with parvalbumin. The FABP from rat skeletal muscle was tested for the presence of Ca^{2+} by atomic absorption spectrophotometry. The protein was found to contain two moles of Ca^{2+} per mole of protein (see Table I) as does parvalbumin (71). Virtually the same result (1.9 mol Ca^{2+}/mol of protein) was obtained with FABP which was first delipidated by passing it through a Lipidex-1000 column at 37 ^{o}C. A comparison of the amino acid compositions of the FABP from rat skeletal muscle with that of parvalbumin (72) from the same animal tissue provides strong evidence for the suggestion that these two proteins are similar, if not identical proteins (see Table II). It is interesting to note that the amino acid composition of heart FABP is very similar to that of skeletal muscle FABP (see Table II) even though their ultraviolet spectra are strikingly different (see Fig. 16).

Table I

Calcium content of FABPs from rat heart and skeletal muscle

FABP	Conc.	Treatment	Ca^{2+} Protein	Ca^{2+} Buffer	Ca^{2+}/Protein[a]
	mg/ml		mg/ml		mol/mol
Skeletal muscle	5.8	delipidated[b]	0.035	<0.001	2.1
	3.0	delipidated	0.019	<0.001	2.2
	4.0	none	0.021	<0.001	1.9
Heart	0.52	none	<0.001	<0.001	0
	1.3	none	0.001	0.0012	0

[a] The calcium contents were determined by atomic absorption spectrophotometry.

[b] Delipidation by passing the FABP through a Lipidex-1000 column at 37°C.

Table II

Amino acid compositions of rat parvalbumin and FABPs

Amino acid	Skeletal muscle FABP	Skeletal muscle parvalbumin[a]	Heart FABP	Liver FABP[b]
Asp	13.1	14	14.4	11
Thr	5	5	5	12
Ser	9	11	9.2	6
Glu	10.1	9	10	17
Pro	ND[c]	0	ND[c]	2
Gly	11	9	9.8	12
Ala	9.3	11	9.3	2
Cys	ND[c]	0	ND[c]	1
Val	5.7	5	5.6	12
Met	3.3	3	4.4	7
Ile	6.2	6	6.9	9
Leu	9.5	9	10.3	6
Tyr	0.4	0	0.6	3
Phe	7	8	6.4	6
His	2.6	2	1.7	2
Lys	14.4	16	17	17
Arg	1.5	1	2.3	2
Trp	ND[c]	0	ND[c]	0

[a] From reference 73 [b] From reference 20 [c] ND. not determined

A comparison of parvalbumin from rabbit skeletal muscle with the FABPs from rat heart and skeletal muscle by isoelectric focusing revealed all three proteins to have acidic pIs (see Fig. 17). The pI of rabbit parvalbumin was found to be 5.5, a value which is identical with the value reported by Capony et al. (74). The pI values for the FABPs from rat skeletal muscle and heart are 5.4 and 5.1, respectively. The value of 5.4 is virtually the same as the value of the 5.3 reported for parvalbumin from rat skeletal muscle (71). I had used for this isoelectric focusing experiment a new preparation of skeletal muscle FABP which contained an impurity that comigrated with rat heart fatty acid binding protein (see Fig. 17). This observation led me to question whether the skeletal muscle FABP consists mainly of parvalbumin with a small contamination of heart FABP. To answer this question, I subjected fatty acid binding protein from skeletal muscle to electrophoresis on a polyacrylamide slab gel. Small sections of the slab gel were stained for protein and scanned (see Fig. 18A). From the main portion of the gel, two sections were cut, which corresponded to the major protein, presumably parvalbumin (section I in Fig. 18A), and to the impurity that comigrated with heart fatty acid binding protein (section II in Fig. 18A). Peak III in Fig. 19A corresponded to a protein impurity which was not present in the purified skeletal muscle FABP. Gel sections I and II were extracted with buffer and the eluted proteins were tested for their capacity to bind oleic acid. Only the protein extracted from gel section I emerged from a Sephadex-75 column with oleic acid bound to it. Hence, the major protein, which by all criteria is identical with parvalbumin, has the capacity to bind fatty acids.

Fig. 17. Isoelectric focusing of FABPs from rat skeletal muscle and heart as well as rabbit parvalbumin on a polyacrylamide slab gel containing ampholine (pH 4-6.5).

Main panel: Absorbance scan of rat skeletal muscle FABP gel after staining with Coomassie brilliant blue and pH(▲) as a function of Rf Inset: Photograph of part of the isoelectric focusing gel. A, Skeletal muscle FABP. B, Rabbit parvalbumin. C, Heart FABP.

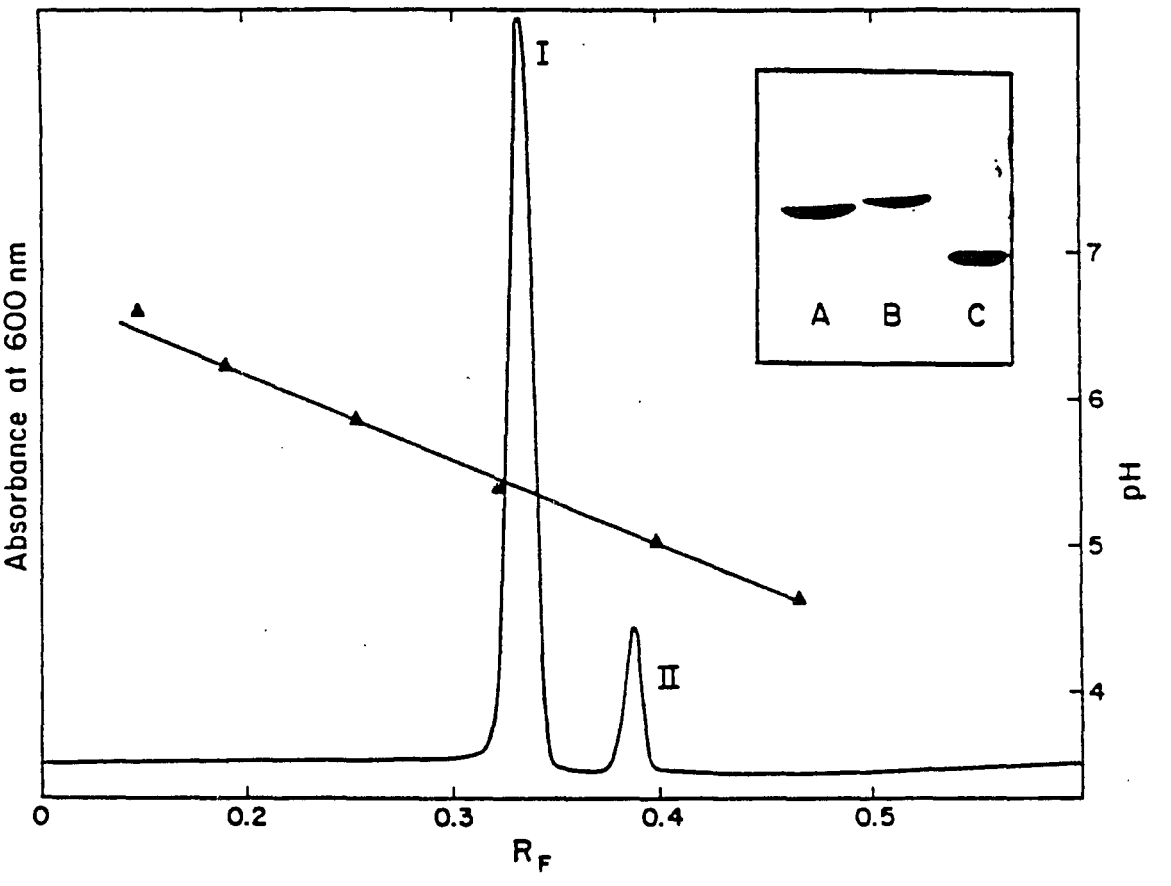

Figure 17

Fig. 18. Purification of rat skeletal muscle FABP by polyacrylamide disc gel electrophoresis and determination of oleic acid binding to purified FABP.

A. Rat skeletal muscle FABP (1 mg) was subjected to electrophoresis on a 6.2% polyacrylamide slab gel. A small section of the gel was stained for protein and scanned. From the main part of the gel two sections corresponding to the major protein (I) and a minor protein (II) were cut. The proteins were extracted from the gel slices with 10 mM KPi (pH 7.4). B. The extracted proteins were charged with 2 nmol of [9,10-^{3}H]oleic acid (0.5 uCi/nmol) and passed through a Sephadex G-75 column (0.63 X 13 cm) equilibrated with 10 mM NaPi (pH 7.4).

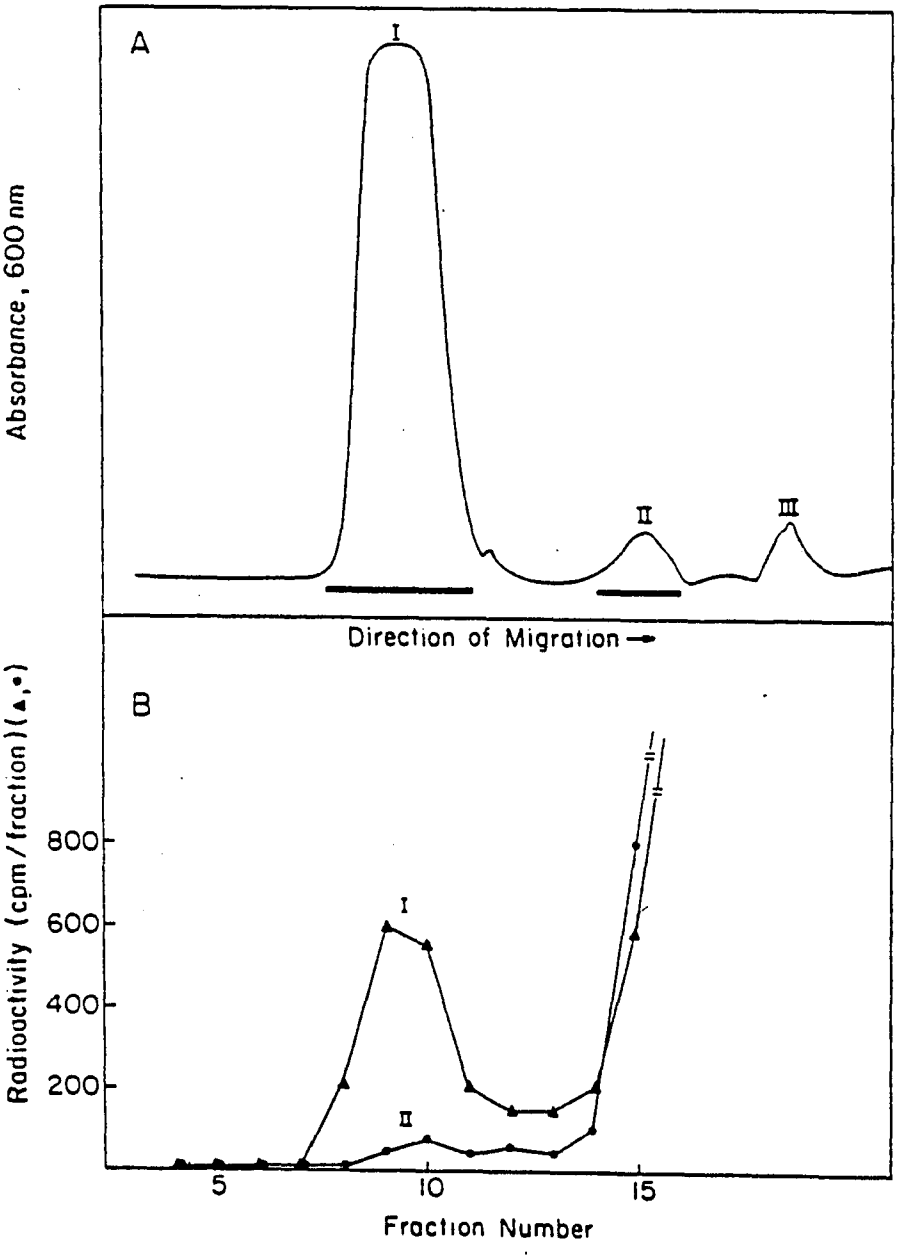
A
I
II
III
Absorbance, 600 nm
Direction of Migration
B
Radioactivity (cpm/fraction) (▲,•)
800
600
400
200
I
II
5
10
15
Fraction Number

Figure 18

Effect of Rat Heart FABP on the Transfer of Oleic Acid Between Membranes.

The effect of FABP on the transfer of fatty acids between membranes was determined by measuring the transfer of [9,10-^{3}H] oleic acid, from a donor membrane to liposomes in the presence or absence of partially purified rat heart FABP (see Fig. 19). The transfer of oleic acid from the donor membrane to liposomes was dependent on the incubation time (see Fig. 20). This process reached an equilibrium after approximately 10 min (Fig. 21). Both in the presence and absence of FABP approximately 40 % of the oleic acid remained bound to the donor membrane. Most the oleic acid leaving the donor membrane was associated with liposomes, while a small percentage of the acid appeared as free fatty acid in solution (see Fig. 20). In the presence of FABP the same amount of oleic acid left the donor membrane. However, one third of the transferred oleic acid was associated with FABP, while two-thirds were bound to liposomes.

Fig. 19. Model system used to study the effect of heart FABP on the transfer of oleate between membranes.

L, phospholipid; FA, fatty acid. For experimental details see "Experimental Procedures".

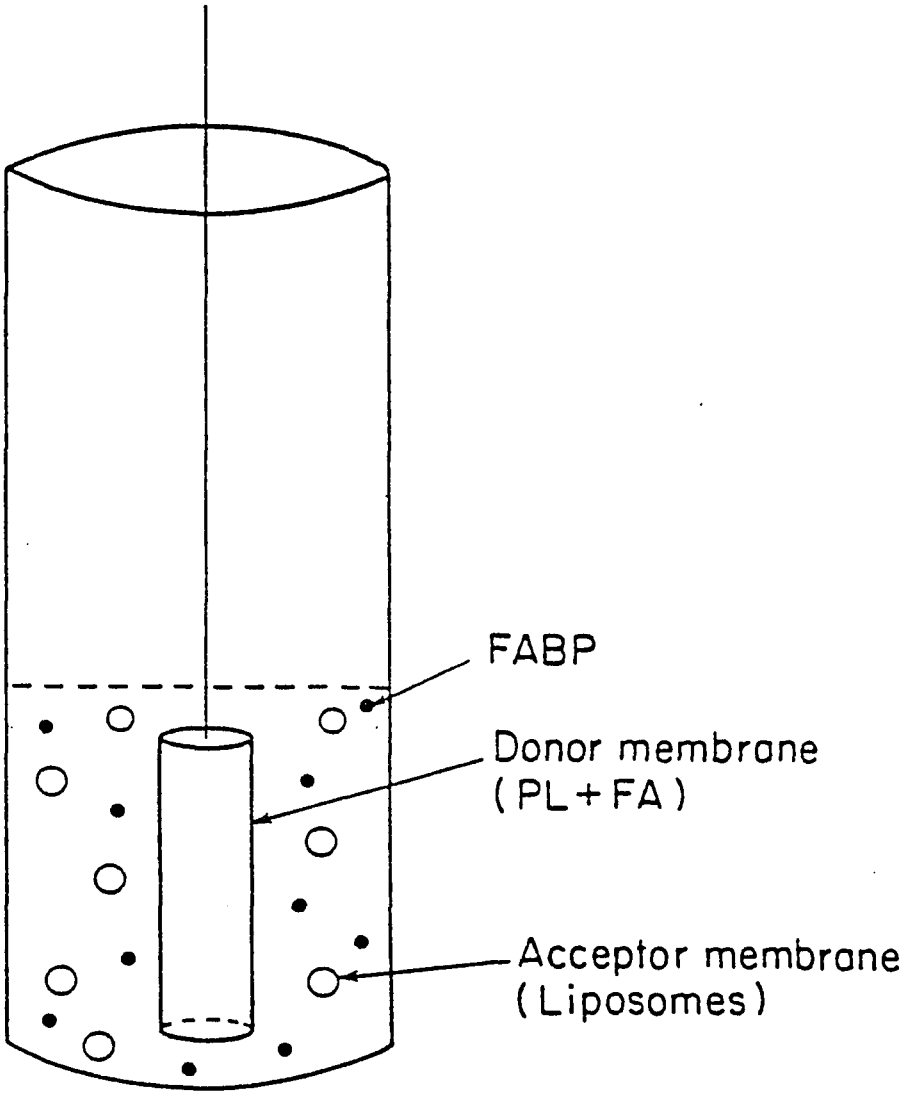

Figure 19

Fig. 20. Separation of liposomes, heart FABP and free fatty acids (FFA) on Sephacryl-S-300.

The incubation mixture contained the donor membrane and A, Liposomes B, liposomes and heart FABP. Distribution of oleic acid after (□) 1 min; (▲) 3 min; (●) 10 min. For experimental details see "Experimental Procedures".

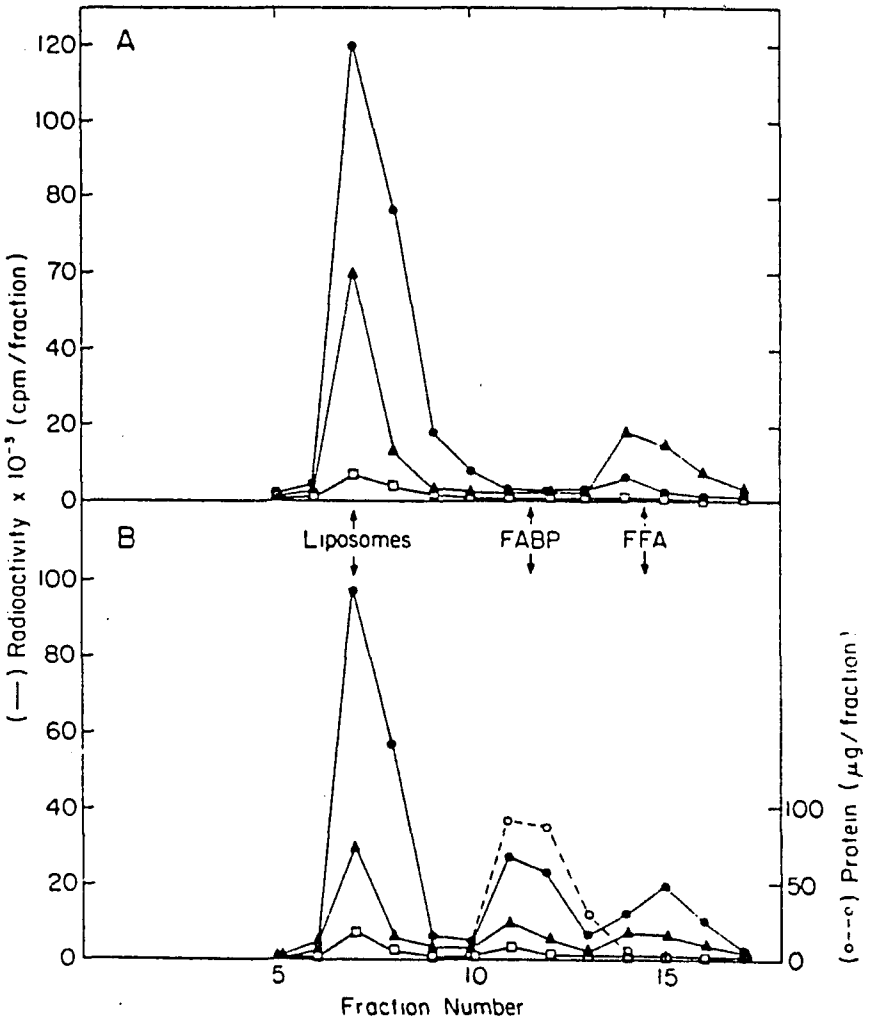
A
B
Liposomes
FABP
FFA
(—) Radioactivity x 10^{-3} (cpm/fraction)
(o--o) Protein (μg/fraction)
Fraction Number
120
100
80
70
40
20
0
60
50
5
10
15

Figure 20

Fig. 21. Kinetics of oleate transfer.

(●) Transfer of oleate between membranes in the absence of FABP; (▲) transfer of oleate between membranes in the presence of heart FABP. (■) transfer of oleate from the donor membrane to heart FABP.

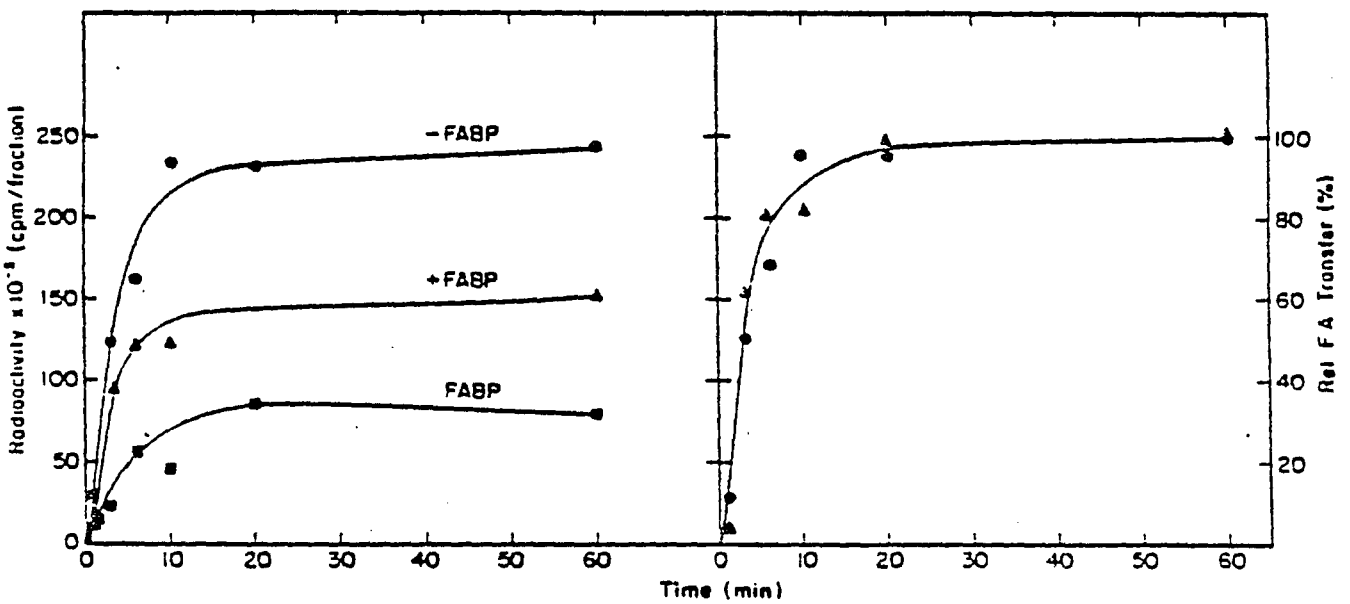
Radioactivity x 10⁻³ (cpm/fraction)
250
200
150
100
50
0
-FABP
+FABP
FABP
0
10
20
30
40
50
60
Time (min)
Rel F A Transfer (%)
100
80
60
40
20

Figure 21

Discussion

Different fatty acid binding proteins (FABPs) of low molecular weight have been identified in various tissues of the same animal. However, their structural relationships have not yet been examined in detail. The results of this study demonstrate that the FABPs from rat liver, heart and skeletal muscle are not identical.

The conclusion that the FABPs of rat liver and heart are not identical is based on four lines of evidence: (a) The two proteins behave differently on polyacrylamide gel electrophoresis under non-denaturing conditions. (b) The two proteins are immunologically unrelated as evidenced by use of antibodies to the FABPs from rat heart and liver. (c) Their amino acid compositions are different. (d) The ultraviolet spectra of the two proteins are different.

The immunological identification of a heart-type FABP in skeletal muscle prompted me to purify this protein. This tissue would provide larger quantities of a FABP from a tissue active in fatty acid degradation, but not in lipogenesis. Although the FABP from rat heart and skeletal muscle are immunologically related and have similar amino acid compositions, they are different proteins. This was demonstrated by their different mobilities on polyacrylamide gel electrophoresis in the presence and absence of SDS and by isoelectric focusing on polyacrylamide gel. In addition, they have different capacities to bind fatty acids before and

after delipidation on Lipidex-1000 and they have different ultraviolet spectra.

This study increases the numbers of different FABPs that have been identified in rat from three to five. One, the liver FABP type is found in liver and intestine, the latter of which contains, in addition, an intestinal specific FABP (61). The hepatic and intestinal FABPs from rat have been sequenced (21,36) and their primary structures show significant homologies. The third and fourth FABPs of rat are the heart (22) and skeletal muscle proteins, respectively. The fifth FABP was isolated from rat brain. It has a low affinity for fatty acids and a pI of 7.2 (12,13). Although the FABP from rat adipose tissue was reported to be similar to that of liver (11), it also may be a tissue-specific protein. This suggestion is based on my inability to detect this FABP with antibodies to rat liver FABP, while I had no difficulty in identifying FABP in rat liver (see Fig.11). Antigens, which cross-reacted with antibodies to heart FABP, were detected in kidney and stomach. If these antigens are FABPs they also may be distinct proteins. What emerges from a number of studies, including this one, is a picture of a group of ubiquitous low molecular weight proteins, all of which can bind fatty acids, but are structurally dissimilar.

This study took a very interesting turn when it became apparent that FABP of rat skeletal muscle contains Ca^{2+} and is related to or

identical with parvalbumin. I abstained from purifying rat parvalbumin because the procedures for purifying rat parvalbumin and FABP are virtually identical and parvalbumin has no distinct biological activity which would permit me to monitor its purification. However, FABP and parvalbumin of rat skeletal muscle are identical by all applied criteria. Rabbit parvalbumin (purchased from Sigma), in contrast to the rat protein, did not bind fatty acids. Although parvalbumin is believed to function in the regulation of fast-twitch skeletal muscle fibers and/or in the regulation of Ca^{2+}-dependent cellular processes (73), no definite proof for its physiological function has been obtained. Hence, both parvalbumin and FABP are proteins without proven biological functions.

Although not proven, it is possible that the FABPs have one or more of the following functions; (a) they may bind fatty acids, fatty acyl-CoA thioesters, and other hydrophobic compounds, thereby preventing these compounds from binding to proteins and membranes in a non-specific and undesired manner (b) they may regulate cellular processes by sensing the concentration of cytosolic ligands, like free fatty acids; (c) they may transport their ligands through the cytosolic compartment; (d) they may assist as acceptors in the transport of their ligands across membranes.

The structural diversity of FABPs from different tissues and the variety of their possible functions will make it difficult to elucidate the

biological roles of these proteins. Kinetic evaluation of oleate transfer between a fixed donor membrane and liposomes acceptors (see Fig.23) demonstrated that heart FABP does not stimulate this process. This observation, as well as the nonidentity of FABPs from different rat tissues, raises doubts about the suggested singular function of these proteins in the cytosolic transport of fatty acids.

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