papers and notes on methodology

Quantitation of hepatic fatty acid-binding proteins by post-chromatographic ligand binding assay

F. D. Morrow[1] and R. J. Martin[2,3]

Department of Foods and Nutrition, University of Georgia, Athens, GA 30602

Abstract A new procedure for the detection and quantitation of small molecular weight cytosolic fatty acid-binding proteins (FABP) in chromatographic fractions is described. Aliquots of the fractionated cytosol are incubated with radiolabeled palmitate and the unbound fatty acid ligand is quickly removed by addition of a dextran-gelatin-coated charcoal suspension. Quantitation of the FABP is accomplished by counting the protein-bound radioactivity in the supernatant fraction after a brief centrifugation step. Validation studies have shown the assay to be linear over a range of 10–40 μg or 20–80 μg of FABP depending on specific activity of [^{14}C]palmitate used. Bovine serum albumin can be included as an external binding protein to correct for the nominal day-to-day variation in the assay system. The assay has been found to give consistent results with a wide variety of buffer salts, ionic strength, and pH, and therefore is compatible with the usual conditions of gel filtration, ion exchange, and affinity chromatography. Compared to detection methods involving prechromatographic addition of bromosulfophthalein or radiolabeled fatty acids to cytosolic proteins, the post-chromatographic binding assay offers the advantage of leaving the bulk of the FABP preparation free of these marker ligands.—**Morrow, F. D., and R. J. Martin.** Quantitation of hepatic fatty acid-binding proteins by post-chromatographic ligand binding assay. *J. Lipid Res.* 1983. **24:** 324–331.

Supplementary key words Z-protein • azo-carcinogen binder II • aminoazodye-binding protein A • bromosulfophthalein • fatty acid transport • bovine serum albumin • fatty acids

Abbreviations: FABP, fatty acid-binding proteins; BSA, bovine serum albumin; PMSF, phenyl methyl sulfonyl fluoride; BEq, binding equivalents; SDS, sodium dodecyl sulfate; BSP, bromosulfophthalein.

[1] Recipient of American Institute of Nutrition Foremost-McKesson Fellowship. Present address: Harvard University School of Public Health, Boston, MA 02115.

[2] Address reprint requests to R. J. Martin, Department of Foods and Nutrition, Dawson Hall, University of Georgia, Athens, GA 30602.

[3] Recipient of NIH Career Development Award AM00818.

Because of their extreme insolubility within the aqueous environment of the cell, long chain fatty acids are present primarily bound to a variety of cytoplasmic proteins. Of particular interest is a series of small molecular weight species (ca. 12,000 daltons) that bind long chain fatty acids with relatively high affinity and are present in nearly all tissues that metabolize significant quantities of these lipids (1–3). Termed fatty acid-binding protein by Ockner and co-workers (1–3), FABP is probably identical to the Z protein originally described by Levi, Gatmaitan, and Arias (4) and Mishkin et al. (5). Ketterer and colleagues (6) have also reported a 14,000-dalton species with similar binding characteristics and named the protein aminoazodye-binding protein A. Experimental evidence suggests that fatty acids in association with FABP may represent the metabolic pool within the cell that supplies the fatty acid utilizing organelles with these lipid substrates (7). However, studies requiring isolation of intracellular FABP have been hampered in part by the lack of a suitable assay for detection and quantitation of these proteins in column chromatographic fractions. To date, most workers have attempted to quantitate tissue levels of FABP by addition of either bromosulfophthalein (4, 8) or radiolabeled fatty acids (9) to a cytosol prepared by high speed centrifugation prior to Sephadex G75 chromatographic separation. The quantity of ligand incorporated into the 12,000-dalton region of the elution profile has been taken as an accurate measure of FABP content of the supernatant proteins. However, due to ligand:column matrix interactions as well as protein:protein competition for available ligand, incorporation of the latter onto the binding protein does not correlate well with the

actual quantity of FABP present (10). The use of radiolabeled fatty acids under conditions of nonequilibrium gel filtration presents particular difficulty since the dextran from which Sephadex is manufactured binds these lipids with high affinity. Further, since the fractionated cytosol is rendered radioactive, subsequent studies on the FABP preparation are difficult or impossible.

A new ligand binding procedure is presented that detects and quantitates soluble fatty acid-binding proteins after completion of the initial chromatographic steps. Since only small aliquots are required for the assay procedure itself, the bulk of the partially purified FABP preparation remains free of exogenously added marker ligands. Although it is not clear what effect the presence of such ligands (e.g., bromosulfophthalein) may have on electrophoretic or kinetic studies of FABP, our own experience has shown that altered isoelectric focusing patterns may result when the latter compound is bound to sites on the FABP molecule.

MATERIALS AND METHODS

Materials

Dextran (clinical grade, average molecular weight 80,700), gelatin (swine skin, Type I, 300 bloom), Tris-hydroxyl-aminomethane, phenylmethyl sulfonyl fluoride (PMSF), bovine serum albumin (fraction V), and bromosulfophthalein (BSP) were all obtained from Sigma Chemical Co., St. Louis, MO. The charcoal (carbon decolorizing alkaline Norit-A) was obtained from Fisher Scientific, Inc. All other chemicals and reagents were analytical grade or equivalent. The Sephadex (fine grade) as well as the chromatograhic column (K26/100) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Radioactive fatty acids were purchased from New England Nuclear Corporation and were of the specific activity indicated in the text.

Isolation of fatty acid binding proteins

Tissue preparation. Liver was chosen as a convenient source of intracellular FABP due to the relatively high concentration of these proteins in this tissue (11). Livers were excised from ad libitum-fed lean male Zucker (fa/f?) rats and immediately rinsed in ice-cold saline to quickly cool the tissue. The tissue was blotted dry, minced into fine pieces with scissors, and homogenized in four volumes of cold buffer by the use of a Brinkman Polytron homogenizer equipped with the PT-10 generator tip. The buffer, pH 7.9, contained 20 mM Tris-chloride, 150 mM KCl, 20 μg/ml PMSF, and 20% (v/v) anhydrous glycerol. All steps were performed at 0–4°C unless otherwise indicated.

Centrifugations. The whole liver homogenate was first spun at 10,000 *g* for 20 min to remove cellular debris and mitochondria. Surface triglyceride was removed by aspiration and the low speed infranatant was then recentrifuged in a Beckman preparative ultracentrifuge at 105,000 *g* for 90 min. The sample tubes were carefully removed from the tube holders and the lipoproteins near the surface were removed by aspiration.

Gel permeation chromatography of liver cytosol fraction. Ten ml of the high speed cytosol was applied to a column of Sephadex G75 (2.6 × 100 cm) using a syringe pump to ensure even sample application. The sample was followed by 5 ml of a 10% (v/v) sucrose-buffer solution to sharpen the lower sample boundary and minimize viscous streaming of cytosol. Fractionation of the supernatant proteins was accomplished using ascending elution at a constant flow rate of 41 ml/hr. The elution buffer was the same as described above except that the PMSF and the KCl were omitted at this step. Ten-min fractions were collected in 13 × 100 mm borosilicate glass tubes and 150-μl aliquots were assayed for fatty acid-binding activity by the procedure described below.

Preparation of assay mixtures

Assay buffers. The buffers used throughout the binding assay were the same as those described above for the elution of the supernatant proteins with two exceptions. In preparation of the labeled fatty acid solution, propylene glycol was found to be superior to glycerol for solubilization of the palmitate and was therefore substituted for this reagent at a reduced concentration (10% v/v). Neither solubilizing agent was found to interfere significantly with the ligand binding assay and they offered the further advantage of preventing aggregation of the small molecular weight FABP. The aggregation of the FABP does not appear to significantly reduce palmitate-binding capacity but can result in the eventual precipitation of these species from solution (12). In preparation of the dextran-gelatin-coated charcoal suspension, neither solubilizing agent was found to be necessary and was routinely omitted.

Preparation of the labeled fatty acid working solution. [^{14}C]Palmitic acid was used throughout the development of this assay due to its chemical stability compared to unsaturated fatty acids, as well as its relative importance in hepatic fatty acid metabolism. Two specific activity preparations were examined (7.6 mCi/mmol and 42.6 mCi/mmol) and compared with regard to the effect on overall assay performance. Although the higher specific activity preparation afforded increased assay

TABLE 1. Composition of assay solutions

[^{14}C]Palmitate Working Solution	Charcoal Suspension
20 mM Tris-Cl, 200 ml	20 mM Tris-Cl, 200 ml
0.5 M NaCl	0.5 M NaCl
Propylene glycol (10% v/v)	pH adjusted to 7.7 (25°C)
pH adjusted to 8.4 (25°C)	1 g Dextran
3.6 μCi [^{14}C]Palmitate[a] (40,000 dpm/ml)	1 g Gelatin
	1 g Powdered carbon

[a] Radiolabeled [^{14}C]palmitate with a specific activity of 7.6 mCi/mmol is recommended for FABP concentrations of 30–120 μg/ml, or 42.6 mCi/mmol for FABP concentrations of 10–40 μg/ml.

sensitivity, the standard curves generated were observed to suffer a decreased linear range. It is suggested that use of the higher specific activity preparations be reserved for samples possessing lower levels of fatty acid-binding species.

The crystalline labeled palmitic acid preparation was converted to the potassium salt by the addition of several drops of saturated KOH and distilled water so that the final activity was approximately 25 μCi/ml. This stock [^{14}C]palmitate was routinely stored at −20°C until needed for preparation of the diluted working solution used in the ligand binding assay procedure. The diluted working solution was most conveniently prepared by placing a beaker (400 ml) inside a larger vessel containing water at a temperature of 50–55°C. This temperature could be maintained and the contents of the inner beaker were constantly agitated by placing the entire set-up on top of a hotplate-magnetic stirrer apparatus. For routine applications where up to 150 samples were to be assayed at one time, 200 ml of the propylene glycol buffer solution was added to the inner vessel. When the 55°C temperature was attained, approximately 3.5 μCi of the stock [^{14}C]palmitate suspension was added and immediately sonicated at maximum power for several minutes to completely solubilize the fatty acid ligand. After sonication, the beaker was returned to the stirred water bath and the activity was checked by removing a 1-ml aliquot from the inner beaker. The standard curves presented were generated from a labeled palmitate solution containing a final activity of 40,000 dpm/ml. Since it is important that each sample tube receive the same amount of radioactivity, the [^{14}C]palmitate solution should be stirred at all times while making these additions. It is also essential that the labeled solution be sonicated thoroughly to ensure complete solubilization of the fatty acid ligand. This is most easily checked by periodically removing aliquots and counting in the liquid scintillation counter.

Preparation of the dextran-gelatin-coated charcoal suspension. The charcoal suspension was prepared immediately before use by mixing 200 ml of ice-cold Tris-Cl, 20 mM, pH 7.7, with 1 g of dextran, 1 g of gelatin, 1 g of the alkaline Norit-A powdered carbon, and 5.8 g of NaCl in a beaker. The suspension was kept at 0°C by placing the beaker inside a larger vessel containing ice. The contents of the inner beaker should be stirred at all times by use of a magnetic stirring apparatus. The mixture should be stirred for approximately 30 min prior to use in order to allow the dextran to swell and adequately coat the charcoal particles. Failure to allow sufficient time for coating of the charcoal with the dextran was observed to result in a slight loss of binding proteins from the post-centrifugation supernatant. Once prepared, the dextran-gelatin-charcoal suspension should be used within 2–3 hr in order to avoid unacceptably high blank values. **Table 1** summarizes the composition of the assay buffers.

Assay of chromatographic fractions for fatty acid binding activity

The assay of up to 150 chromatographic fractions can be completed within 90 min by arranging the hotplate-stirrers, vortex mixer, water bath, and ice bath in an "assembly line" fashion atop the workbench. Pipetting of [^{14}C]palmitate solution and dextran-gelatin-coated charcoal suspension is most conveniently accomplished by the use of a hand-held pipettor with disposable plastic tips. Pipetting of the charcoal suspension may require that the orifice of the plastic tip be slightly enlarged to avoid clogging.

Incubation of fraction aliquots with fatty acid ligand. One-ml aliquots of the labeled palmitate working solution (40,000 dpm/ml) are pipetted into disposable 12 × 75 mm glass culture tubes. Tube racks of the type commonly used for radioimmunoassay were found to work quite satisfactorily. During this step the racks should be partially immersed in a thermostatically regulated water bath set for 45°C. Fraction aliquots containing 20–80 μg of FABP (10–40 μg of FABP for the high specific activity preparation) are then added in a *total volume of 500 μl*. Each sample is vortexed briefly and returned to the water bath. The elevated temperature was found to accelerate uptake of the palmitate label onto the binding sites of the proteins and complete equilibration was observed within 2 min of sample addition. However, for convenience, 10-min incubation times were routinely used for this step of the procedure.

Stabilization of bound ligand. To minimize loss of palmitate label from binding sites on the proteins during subsequent steps, the tubes are immersed in an ice bath for 10 min to stabilize the bound ligands.

Removal of unbound fatty acid and centrifugation. One-ml aliquots of the dextran-gelatin-coated charcoal mixture are removed from the *stirring* suspension and quickly added to each tube. The latter are then vortexed briefly (approximately 1 sec) and immediately centri-

fuged. Centrifugation of the 12 × 75 mm tubes was conveniently accomplished by use of a Dupont-Sorvall radioimmunoassay swinging bucket rotor (Model HS-4) with a capacity of 96 tubes. The centrifugation step (5 min, 3200 *g*) is performed at 4°C in a refrigerated centrifuge. Exposure of the binding proteins to the unsedimented charcoal suspension for periods greater than 3–4 min was observed to reduce net palmitate uptake. To minimize the time required for completion of the charcoal suspension, we found it helpful to perform multiple centrifugation runs where no more than 40 samples were processed at one time. Failure to work quickly at this step could otherwise result in loss of assay sensitivity and linearity.

Determination of sample fatty acid-binding activities. Removal of sample tubes from the centrifuge should be performed carefully since agitation of the pelleted charcoal results in high blank values and poor sample replication. Counting of protein-bound radioactivity is accomplished by removing 500-μl aliquots from each tube and counting with an aqueous immiscible scintillation fluid. Blank values for evaluating the efficiency of charcoal absorption of unbound fatty acid are determined by substituting the fraction samples with the glycerol containing elution buffer. Blank values should be determined in quadruplicate and the average should be subtracted from each sample's gross cpm. Typical blank values are 300–500 cpm for the high specific activity palmitate, or 400–500 cpm for the low specific activity preparation.

Use of bovine serum albumin as a reference binding protein

Inter-assay variation in the ligand binding procedure was found to be nominal and was readily adjusted by including an external standard binding protein. Bovine serum albumin (Sigma Chemical Co., Fraction V) was routinely used for this purpose due to the availability of relatively pure preparations of this protein. Comparison of regular "Fraction V" preparation with either "essentially fatty acid-free" type, or with serum albumin from several other species (rat, human, porcine, chicken) showed no significant differences in palmitate-binding capacity. Label incorporation was found to be linear up to approximately 50% of the available palmitate bound. **Fig. 1** illustrates a typical standard curve using bovine serum albumin over the range of 10–40 μg[4] per assay tube. Reference serum albumin solutions (20–80 μg/ml) were prepared daily in glycerol-elution buffer since either freezing or storage at 4°C for several days resulted in diminished fatty acid-binding capacity. Linearity and sensitivity of the ligand binding assay for either whole liver cytosol fraction or partially purified FABP preparations were found to be similar to that observed for the BSA reference solutions. It was therefore useful to express the fatty acid-binding capacity of unknown samples in terms of "binding equivalents." The latter is defined in our assay system as the amount of [^{14}C]palmitate bound per 10 μg of regular Fraction V bovine serum albumin reference solution. **Table 2** presents a comparison of partially purified FABP and serum albumin in terms of fatty acid-binding equivalents. When expressed in this manner the fatty acid-binding capacity of partially purified FABP preparations can be more readily compared between chromatographic steps as well as between different laboratories.

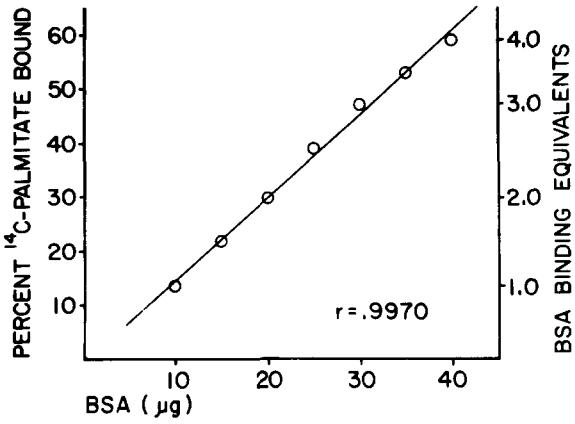

Fig. 1. Standard curve for bovine serum albumin using the ligand binding procedure described in the text. The assay response for BSA is linear over the range of 10–60% of [^{14}C]palmitate bound. The above curve was generated using the 7.6 mCi/mmol palmitate preparation at 40,000 dpm/ml.

RESULTS AND DISCUSSION

Comparison of Sephadex G75 elution profiles

Liver cytosol was prepared as described above and used to compare the chromatogram generated by the new method to that obtained by precolumn addition of either radiolabeled fatty acid or bromosulfophthalein (BSP). The elution profile shown in **Fig. 2** is that obtained when [^{14}C]palmitic acid is added prior to sample application as described by Katongole and March (9). **Fig. 3** represents the same cytosolic preparation into which BSP (600 μM) was added prior to elution. BSP was detected spectrophotometrically at 580 mm after alkalinization with 1 N NAOH. Fatty acid-binding ac-

[4] Use of the high specific activity [^{14}C]palmitate preparation (42.6 mCi/mmol) allows the assay sensitivity to be increased approximately fourfold. The reference serum albumin solutions should therefore be adjusted to give a final concentration of 2.5 to 12.5 μg BSA per assay tube.

TABLE 2. Comparison of bovine serum albumin and partially purified fatty acid binding protein[a] in terms of binding equivalents (BEq)[b]

BSA (μg)	Percent Palmitate Bound	Theoretical BEq	Calculated BEq/mg Protein	FABP Preparation (μg)[c]	Percent Palmitate Bound	Calculated BEq/mg Protein
10	13.64	1.0	93.6	41.6	9.43	16.41
20	30.11	2.0	101.3	83.2	18.05	14.89
30	47.45	3.0	99.1	124.8	26.24	14.25
40	59.20	4.0	98.8	166.4	31.72	12.77

[a] Tubes 42–52 of the Sephadex G75 gel-filtered liver cytosol.
[b] One BSA binding equivalent is equal to the fatty acid binding capacity of 10 μg of regular fraction V albumin in the assay described, using the low specific activity [^{14}C]palmitate label (7.6 mCi/mmol).
[c] Protein determined by the method of Lowry et al. (Ref. 20).

tivity (BEq/ml) was also assayed in the fractionated cytosol of both chromatographic runs using the ligand-binding procedure described.

Fig. 2 shows that the new procedure results in a chromatogram nearly identical to that obtained using pre-column addition of radiolabeled fatty acid. A variety of larger molecular weight cytoplasmic proteins capable of binding long chain fatty acids was also detected and supports earlier studies in which this ligand was added prior to fractionization (13–15). Assay of unfractionated liver cytosol using the ligand-binding procedure thus reveals *total* cytoplasmic binding capacity of which 40–60% is accounted for by the 12,000-dalton FABP species. The co-elution (Fig. 3) of a fatty acid-binding species and a BSP-binding species in the M_r = 12,000-dalton region of the profile also supports earlier studies (2, 3) which suggested that FABP and Z protein may in fact be the same molecule. If they are indeed the same protein, it is evident that the BSP-binding capacity of this protein is relatively minor in comparison to the larger molecular weight BSP-binding proteins that elute earlier in the chromatogram. In contrast, the fatty acid-binding capacity of the 12,000-dalton protein represents a major portion of the total protein bound fatty acid ligand. It is also of interest that for proteins larger in molecular weight than M_r = 12,000, the elution profiles obtained using BSP (pre-column addition) or [^{14}C]palmitate (post-column binding assay) are essentially different. This suggests that at least some proteins capable of binding the one hydrophobic anion do not necessarily bind the other to a significant extent. The profile presented in Fig. 3 indicates that FABP does indeed bind both small organic anions and long chain

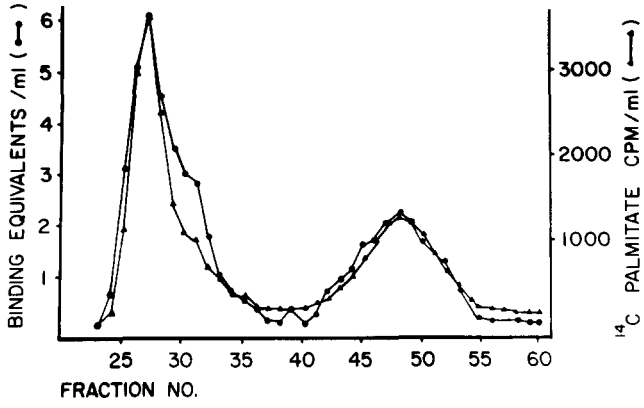

Fig. 2. Comparison of Sephadex G75 elution profiles. Liver cytosol (10 ml) containing [^{14}C]palmitic acid (175,000 dpm/ml) was applied to a calibrated column (2.5 × 95 cm) and recovered using ascending elution. Fraction aliquots were counted directly or assayed for fatty acid binding activity by the post-chromatographic procedure described. The new procedure results in a chromatogram nearly identical to that obtained by pre-column addition of ^{14}C-labeled fatty acid, but leaves the bulk of the fractionated sample free of radioactivity or other exogenously added ligands.

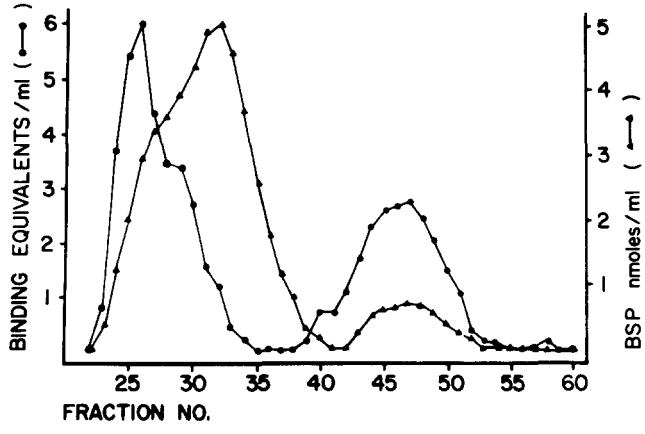

Fig. 3. Bromosulfophthalein Sephadex G75 elution profile. BSP (600 μM) was added to liver cytosol (10 ml) and the sample was eluted from a calibrated column (2.5 × 95 cm) using ascending elution. Fraction aliquots were assayed for BSP by measuring the absorbance at 580 nm after alkalinization with 1 N NaOH. Fatty acid binding activity (BEq/ml) was quantitated by the new procedure described. The profiles reveal that while the protein(s) in the 12,000-dalton region of the chromatogram represent a minor portion of the total cytoplasmic BSP binding capacity, they represent 40–60% of the total cytoplasmic fatty acid binding capacity.

fatty acids but that the binding of the latter may be of greater quantitative significance when the entire ligand-binding profile is examined. One could not conclude from these studies that the apparent preferential binding of long chain fatty acids in vitro is of physiological significance in the intact liver cell.

FABP dose–response curve

Fig. 4 presents a dose–response curve in which the partially purified FABP preparation from the gel filtration step was assayed by the new ligand-binding procedure. Since FABP purified to homogeneity was not available, this preparation also included substantial quantities of non-FABP species. Kinetic studies performed by Warner and Neims (12) suggest that the content of bona fide fatty acid-binding species in this preparation is approximately 50% of the total protein. Our own estimate based on densitometric scans of native disc gel electrophoretograms suggests a figure closer to 35% of the total protein (data not presented). Assuming that these estimates are reasonable, we can approximate from the data presented in Table 2 that the specific activity of binding of purified FABP using the new procedure is in the range of 30–40 BEq/mg. This range is approximately one-third that of the BSA external standard (100 BEq/mg). Using the functional expression of BEq/mg protein as a measure of specific activity of binding, we have been able to conveniently estimate recovery and relative purification of FABP in a manner similar to that used by investigators during the isolation of enzymatic proteins.

Close examination of the dose–response curves generated by BSA and FABP revealed that differences between these two binding species was not, however, limited to the specific activity of binding. Whereas the standard curves generated by serum albumin were found to be linear up to 50% of the available palmitate bound, many FABP preparations were observed to generate dose–response curves that were distinctly sigmoidal in character when the protein-bound palmitate exceeded 30% of the total. Although formal analysis of these data was not attempted, the results do suggest that the kinetics of fatty acid binding between the two proteins is quite different if the total palmitate concentration is varied over a similar range of values. For the purpose of quantitation of FABP in chromatographic fractions, a sample volume should be used that falls within the linear portion of the standard curve.

Assay performance

Validation studies were designed to compare assay performance of the post-column ligand-binding procedure to that obtained by pre-column addition of radio-

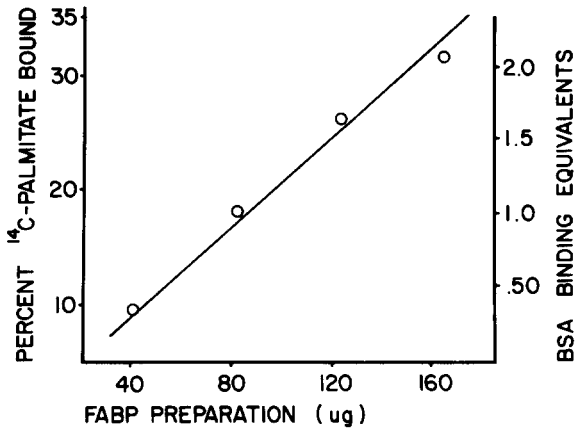

Fig. 4. Dose–response curve for partially purified FABP. Fractions 42–52 from the Sephadex G75 step were pooled and used to examine assay performance. Assay response for these small molecular weight binding species was observed to be linear up to about 35% of the available palmitate bound. Assuming the relative purity of this preparation to be approximately 50% (see text), it is possible to estimate the specific binding activity of pure FABP to be in the range of 30–40 BEq/mg protein, approximately one-third of the bovine serum albumin external standard.

labeled palmitic acid. Comparisons were not made using BSP as a marker ligand due to the relatively low sensitivity afforded by this colorimetric procedure. Liver cytosol was prepared as described under Methods and pooled to allow for quantitation of FABP levels on 6 consecutive days using both procedures. [^{14}C]Palmitic acid (42.6 mCi/mmol) was added to the whole cytosol fraction to a final activity of 175,000 dpm/ml using the procedure described by Katongole and March (9). Ten ml of this radiolabeled sample was then applied to the Sephadex G75 column and eluted as described under Methods. One-ml aliquots were removed from each fraction and counted directly in the scintillation counter. Two-hundred microliter aliquots were also assayed using the post-column assay. The results are tabulated in **Table 3.** The pre-column method resulted in an inter-assay coefficient of variation (C.V.) of 7.9% while that of the ligand binding procedure averaged 5.1%. Eight replicate samples were also assayed on run number 6 using the new procedure and resulted in an intra-assay C.V. of 2.2%. An intra-assay C.V. was not determined on the pre-chromatographic procedure since this would require a system of tandem gel filtration columns. These data support our contention that quantitation of binding proteins by addition of a marker ligand is more reliably accomplished by the use of a post-chromatographic assay procedure. Although a number of factors may contribute to this phenomenon, elution of protein-ligand complexes under conditions of nonequilibrium gel filtration would be expected to contribute to a variable recovery of the marker ligand in the peak of interest.

TABLE 3. Comparison of assay performance

Run No.	Pre-column Method	Post-column Ligand Binding Assay
1	55289 CPM[a]	253.8 BEq[b]
2	57918	250.0
3	63749	222.4
4	68917	258.2
5	64542	251.4
6	63197	248.8
C.V.	7.86%	5.14%

[a] Total CPM incorporated into the M_r = 12,000 FABP peak.
[b] Total binding equivalents (BEq) in the FABP peak as measured by the new method.

Pooled liver cytosol was assayed for FABP levels on 6 consecutive days by both the pre-chromatographic and the post-chromatographic ligand binding assay described. The new procedure was shown to result in a lower coefficient of variation (C.V.) than can be attained by addition of marker ligand prior to fractionization of the cytosolic starting material.

This is of particular concern when the time required for elution is lengthy, or if the chromatographic matrix itself possesses an affinity for the ligand used. In our studies, recovery of radiolabeled fatty acids from the gel filtration step rarely exceeded 60% of that applied to the column and suggests that matrix-ligand interactions probably contribute to loss of marker fatty acids from the binding proteins during elution.

Assay constraints

Validation studies examining the performance of the newly described ligand binding assay reveal a considerable improvement in the quantitation of fatty acid-binding proteins in chromatographic fractions when compared to other nonimmunological methods. However, the new assay requires an initial chromatographic step to separate the small molecular weight FABP from other binding species and thus the assay of large numbers of tissue samples may not be practical. In this instance, quantitation of FABP by immunological procedures such as radioimmunoassay (10) or quantitative radial immunodiffusion (2) would prove more satisfactory. The latter procedures, of course, presume the availability of precipitating antibodies reactive to all forms of FABP that may be present in the unchromatographed sample. Molecular heterogeneity of FABP in liver has been reported (16, 17) and results of our own studies suggest that at least one hepatic form is not highly antigenic. Further, immunological nonidentity among FABP from liver and adipose tissue (18) suggests that antisera useful in quantitating FABP from one tissue may not give valid estimates in another tissue. Investigations similar in nature to those recently reported by Trulzsch and Arias (19) may reveal that "FABP" is in fact a heterogeneous group of proteins, each of which may have different affinities for the various long chain fatty acids. If such is the case, the procedure reported here represents a functional quantitation of the cytoplasmic fatty acid-binding activity which is contributed by these small molecular weight proteins.

A second consideration in addition to sample numbers is the presence of agents in the elution buffers that may interfere with the ligand-binding assay procedure itself. The presence of non-ionic detergents such as Triton-X 100, or of sodium dodecyl sulfate, was found to markedly interfere with the ability of the charcoal suspension to remove unbound [^{14}C]palmitate ligand. High concentrations of chaotropic salts, on the other hand, reduced label uptake onto the binding protein without affecting blank values. The use of such agents would require their substantial removal prior to assay by the new procedure.

SUMMARY

The ligand-binding assay procedure described was developed primarily to facilitate detection and quantitation of small molecular weight FABP in column chromatographic fractions. The binding of [^{14}C]palmitate by FABP, as well as other larger molecular weight species, was found to occur over a wide range of pH and ionic strength. With the notable exception of certain commonly used detergents, very few agents were discovered that interfered with either binding of fatty acid by the proteins or with the ability of the dextran-gelatin-coated charcoal suspension to remove unbound fatty acid ligand. Thus, the assay is compatible with the detection of FABP under the usual conditions of gel filtration, ion exchange, and affinity chromatography. Validation studies showed that the quantitation of FABP by new post-chromatographic ligand binding procedure offers greater assay sensitivity and less inter-assay variation than can be attained by addition of marker ligands to supernatant proteins prior to chromatographic separation. Assay standardization is readily accomplished by including bovine serum albumin as a reference binding protein and allows the fatty acid-binding activity of unknown samples to be expressed in terms of "BSA-binding equivalents." The new ligand binding assay is expected to facilitate studies of FABP requiring the use of chromatographic isolation and purification procedures.

The authors gratefully acknowledge the advice of Mr. J. H. Gahagan in the design and analysis of the validation studies and in the skilled assistance of Ms. Alayne Makula and Ms. Christina Stevens for the graphics. Supported in part by NIH

Grant HD-12947 and a grant from the American Diabetes Association.

Manuscript received 18 September 1981 and in revised form 12 October 1982.

REFERENCES

1. Ockner, R. K. 1972. A binding protein for fatty acids in cytosol of intestinal mucosa, liver, and other tissues. *J. Clin. Invest.* **51:** 70a (Abstract).
2. Ockner, R. K., and J. A. Manning. 1974. Fatty acid binding protein in small intestine: identification, isolation, and evidence for its role in cellular fatty acid transport. *J. Clin. Invest.* **54:** 326–337.
3. Ockner, R. K., and J. A. Manning. 1976. Fatty acid binding protein: role in esterification of absorbed long chain fatty acid in rat intestine. *J. Clin. Invest.* **58:** 632–641.
4. Levi, A. J., Z. Gatmaitan, and I. M. Arias. 1969. Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein, and other anions. *J. Clin. Invest.* **48:** 2156–2167.
5. Mishkin, S., L. Stein, Z. Gatmaitan, and I. M. Arias. 1972. The binding of fatty acids to cytoplasmic proteins: binding to Z-protein in liver and other tissues of the rat. *Biochem. Biophys. Res. Commun.* **47:** 997–1003.
6. Ketterer, B., E. Tipping, J. F. Hackney, and D. Bede. 1976. A low molecular weight protein from rat liver that resembles ligandin in its binding properties. *Biochem. J.* **155:** 511–521.
7. Fournier, N., M. Geoffroy, and J. Deshusses. 1978. Purification and characterization of a long chain fatty acid binding protein supplying the mitochondrial *B*-oxidative system in the heart. *Biochim. Biophys. Acta.* **533:** 457–464.
8. Renaud, G., A. Foliot, and R. Infante. 1978. Increased uptake of fatty acids by the isolated rat liver after raising the fatty acid binding concentration with clofibrate. *Biochem. Biophys. Res. Commun.* **80:** 327–334.
9. Katongole, J. B. D., and B. E. March. 1980. Fat utilization in relation to intestinal fatty acid binding protein and bile salts in chicks of different ages and different genetic sources. *Poultry Sci.* **59:** 819–827.
10. Stein, L. B., S. Mishkin, G. Fleischner, Z. Gatmaitan, and I. M. Arias. 1976. Effect of fasting on hepatic ligandin, Z protein, and organic anion transfer from plasma in rats. *Am. J. Physiol.* **231:** 1371–1376.
11. Fleischner, G., D. K. F. Meijer, W. G. Levine, Z. Gatmaitan, R. Gluck, and I. M. Arias. 1975. Effect of hypolipidemic drugs, nafenopin and clofibrate, on the concentration of ligandin and Z protein in rat liver. *Biochem. Biophys. Res. Commun.* **67:** 1401–1407.
12. Warner, M., and A. H. Neims. 1975. Studies on Z-fraction. I. Isolation and partial characterization of low molecular weight ligand binding protein from rat hepatic cytosol. *Can. J. Physiol. Pharmacol.* **53:** 493–500.
13. Suzue, G., and Y. L. Marcel. 1975. Studies on the fatty acid-binding proteins in cytosol of rat liver. *Can. J. Biochem.* **53:** 804–809.
14. Lunzer, M. A., J. A. Manning, and R. K. Ockner. 1977. Inhibition of rat liver acetyl coenzyme A carboxylase by long chain acyl coenzyme A and fatty acid. *J. Biol. Chem.* **252:** 5483–5487.
15. Ockner, R. K., N. Lysenka, J. A. Manning, S. E. Monroe, and D. A. Bennett. 1980. Sex steroid modulation in fatty acid utilization and fatty acid binding concentration in rat liver. *J. Clin. Invest.* **65:** 1013–1023.
16. Rustow, B., J. Nodi, D. Kunze, G. Reichmann, and E. Egger. 1978. Specific binding of saturated and unsaturated fatty acids on the Z-protein of rat liver cytosol. *FEBS Lett.* **95:** 225–228.
17. Morrow, F. D., and R. J. Martin. 1980. Isolation of hepatic fatty acid binding components. *Federation Proc.* **39:** 1124 (Abstract).
18. Riaz-ul-Haq, E. Shrago, and B. Ketterer. 1981. Isolation of fatty acid binding protein from rat and human adipose tissue. *Federation Proc.* **40:** 336 (Abstract).
19. Trulzsch, D., and I. M. Arias. 1981. Z Protein: isolation and characterization of multiple forms in rat liver cytosol. *Arch. Biochem. Biophys.* **209:** 433–440.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.