MATERIALS AND METHODS

Materials

1. Clinical material

Human placental and fetal tissues (from 5th to 30th weeks of gestation) were collected from patients undergoing legal abortion via hysterotomy according to Medical Termination of Pregnancy (MTP) Act from the Department of Obstetrics and Gynecology, National Medical College and Hospital, Calcutta. Placentas and fetuses of gestational age 30 to 40 weeks were collected as still born from different MTP clinics in and around Calcutta. All the mothers were healthy and aged between 18 and 26 years. In each case, more or less similar nutritional status of the mothers was maintained as much as possible. The fetuses and placentas were placed on ice immediately after operation. Gestational age has been calculated from the period of amenorrhea and by weight and crown-rump length of the fetus (279).

Fetuses were then dissected immediately for excision of liver. Placental tissues were carefully separated from the membrane. Both the placental and fetal liver tissues were then divided into two equal portions, one of which was washed with ice cold 0.9% saline and other with ice-cold 0.25 (M) sucrose solution to make the tissues free from contaminating blood as much as practicable and kept at 0-4^{0}C until use (always within 45 minutes after operation). The saline-washed portion was used for purification of FABP and the sucrose-washed portion for G6PD activity.

Human placentas and livers obtained at different stages of pre-natal development were grouped according to the differences in the gestational period of 5 weeks.

2. Common laboratory reagents

Common laboratory chemicals used in the present experiments were of analytical grade and were purchased from E. Merck, B.D.H., Glaxo, S.R.L., S.D. Fine Chemicals and Saravai M. Chemicals.

3. Fine chemicals

Fine chemicals, e.g. 12-(9-anthroyl)stearic acid, oleic acid, palmitcyl CoA (PAL-CoA), glucose-6-phosphate (G6P), NADP, α-lactalbumin, soybean trypsin inhibitor, trypsinogen, carbonic anhydrase, cytochrome c, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), Tris (hydroxymethyl)amino methane (Tris), Sephadex G-50, DEAE-cellulose, agarose and 1-ethyl-3-(-3-dimethylaminopropyl)carbodimide (EDAC) were purchased from Sigma Chemicals Company, St. Louis, MO, USA. Standard methyl esters of fatty acids for gas-chromatography were obtained from E. Merck, Darmstadt, West Germany. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Difco Chemicals, Detroit, MI, USA. Sephacryl S-200 was a gift from Prof. F. Spener, University of Münster, West Germany.

4. Buffers

Buffers were prepared according to the methods as described in 'Methods in Enzymology'.

Methods

1. Isolation and purification of FABP

FABP from human placenta and fetal liver was purified by modification of the method of Haunerland et al. (280).

A. Tissue processing :

Cross sectional pieces of saline-washed placentas and fetal liver tissues were chopped and homogenized in ice cold 10 mM Tris-HCl buffer, pH 8.5, using an all glass Potter-Elvehjem homogenizer. The homogenate was then strained through one layer of adsorbent gauze to remove the large fragments of the tissues.

B. Preparation of cytosol :

The homogenate was centrifuged at 36,000 x g for 30 minutes and the supernatant was again spun at 105,000 x g for 1 hour. The cytosol thus obtained was heated at 50^0C for 20 minutes and centrifuged at 36,000 x g for 30 minutes. The supernatant was divided in two equal parts and one of these was shaken vigorously with 25% butanol for 1 minute and centrifuged immediately at 36,000 x g for 30 minutes. This supernatant was termed as 'delipidated cytosol' and the other one as 'native cytosol'. These two cytosols were lyophilized and used for further experiments.

C. Gel chromatography in Sephacryl S-200 :

Lyophilized delipidated cytosol was dissolved in minimum volume of 0.01 M Tris-HCl, pH 8.5 and incubated for 30 minutes at 0^0C with 12-(9-anthroyl)stearic acid, dissolved in butanol. This mixture was then chromatographed on a Sephacryl S-200 column (2.5 x 80 cm, flow rate, 20 ml/hour), eluted with the same buffer. Readings were taken at 280 nm for protein and at 364 nm for 12-(9-anthroyl) stearic acid in a Hitachi spectrophotometer (Model No. 200-20). Fractions having 12-(9-anthroyl)stearic acid binding ability were pooled, lyophilized and used for ion exchange chromatography.

Lyophilized native cytosol was also chromatographed as described above, without any prior incubation with 12-(9-anthroyl)stearic acid. Readings were taken at 280 nm for protein in the above mentioned spectrophotometer and fractions corresponding to those having fatty acid binding ability in case of delipidated cytosol, were pooled, lyophilized and used for further studies.

D. Gel filtration on Sephadex G-50 :

Dried powder of the native sample after step C was dissolved in minimum volume of 0.01 M Tris-HCl, pH 8.5 and chromatographed on a Sephadex G-50 column (1.25 x 80 cm, flow rate, 20 ml/hour),

equilibrated with the same buffer. Fractions of 2 ml each were collected. Readings were taken at 280 nm for protein. Fractions showing protein peaks were tested for the presence of endogenous fatty acids. Lipids were extracted from the portions of each peak according to the method of Folch et al. (281) and individual lipids were identified by TLC. Fractions showing presence of endogenous fatty acids were pooled and lyophilized.

E. Ion-exchange chromatography on DEAE-cellulose :

Lyophilized powder of butanol treated sample after step C and of native sample after step D were dissolved separately in 0.01 M Tris-HCl, pH 8.5, and each were charged to DEAE-cellulose column (1.5 x 10 cm, flow rate, 25 ml/hour). Elution was achieved by (a) 0.01 M Tris-HCl, pH 8.5; volume per tube, 2 ml and (b) a linear gradient of NaCl up to 0.3 M in 0.01 M Tris-HCl, pH 8.5; volume per tube, 2 ml. Readings were taken at 280 nm for protein (in both the cases) and at 334 nm for 12-(9-anthroyl)stearic acid (in case of butanol treated sample). Fractions corresponding to peaks were pooled, lyophilized and dialyzed overnight against cold distilled water. These were then used for subsequent studies

2. Characterization of FABP

A. Lipid extraction :

Lipid extraction and purification were carried out according to the method of Folch et al. (281). Dialyzed native protein samples after DEAE cellulose chromatography were treated twice with 19 volume per gm of chloroform : methanol (2:1, v/v) and twice with chloroform : methanol (1:2, v/v). The methanolic chloroform extracts were combined and washed with 1/5 volume of 0.58% NaCl to free it from contaminants. The solvent was removed in a vacuum rotary evaporator at a temperature not more than 40^{0}C. The residue was dissolved in hexane and known aliquots of the purified lipid were used for the following studies. For extraction of covalently

bound fatty acids, protein samples were extracted thrice with chloroform : methanol (1:1) and then hydrolyzed in 5.7 M HCl at 110^{0}C for 24 hours under vacuum. Free fatty acids were extracted from the hydrolyzate as described above.

B. Identification of individual lipids with the help of thin layer chromatography :

Individual lipids were isolated by one-dimensional TLC. Plates (20 x 22 cm) of 0.25 mm thickness were prepared with Silica gel 60 slurry. Portions of lipid extracts were spotted on to the plates and developed in petroleum-ether : diethyl ether : glacial acetic acid :: 80:20:1. The lipids spots were located with iodine vapour and etched round with a fine needle. Appropriate zones were identified by means of standards and were extracted from the chromatogram.

C. Analysis of fatty acids with the help of gas liquid chromatography (GLC) :

Fatty acid methyl esters were prepared by heating 2-4 mg of fatty acids, dissolved in chloroform, with 15-20 ml of a mixture of absolute methanol : benzene : concentrated sulfuric acid :: 86:10:4 for about 2 hours at 80-90^{0}C (maintained by placing the container of the reaction mixture in a water bath) under a current of nitrogen gas (282). After cooling, the volume of methanol was reduced under vacuum and then 3-4 ml of water was added followed by the addition of a drop of methyl orange indicator. The fatty acid methyl esters were extracted from the pinkish aqueous layer with five 1 ml portions of hexane. The combined extracts were dried over fused anhydrous sodium sulfate and brought to proper concentration. The fatty acid methyl esters were separated and quantitated by GLC on a 6 ft x $\frac{1}{8}$ inch column of 10% diethylene glycol succinate on 100-120 chromosorb WHP using a Hewlett Packard Chromatograph, equipped with flame ionization detector,

and maintained with 20 lb pressure of nitrogen gas. Temperature programming was carried out from 120^0-200^0C. Pure methyl esters were used as standards. Assignments for each peak were obtained from a semi logarithmic plot of relative retention time versus chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. Peak areas were determined by multiplying the height by the width (at half height). The relative per cent of each fatty acid methyl ester was determined by dividing the area under individual peaks by the total peak area.

D. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out both under denaturing and non-denaturing conditions. For PAGE in the presence of SDS, method of Laemmli (283) was followed. The protein samples were incubated for 10 minutes at room temperature with 2% SDS and 5% β-MET in 0.0625 M Tris-HCl, pH 6.8, and 10% glycerol and bromophenol blue was used as the tracking dye. The separating gel used usually contained 10% acrylamide (unless specified otherwise) in 375 mM Tris-HCl, pH 8.6 and 0.1% SDS and the stacking gel contained 5% acrylamide in 125 mM Tris-HCl, pH 6.8 and 0.1% SDS. The gels were polymerized by the addition of TEMED and ammonium persulfate. The electrode buffer contained 25 mM Tris, 192 mM glycine, pH 8.3 and 0.1% SDS. The electrophoresis was performed with a current of 4 mA per tube from cathode to anode until the dye front had nearly reached the bottom of the gel. The non-denaturing gel was run essentially in the same way in the absence of SDS.

After electrophoresis the gels were removed from the tubes and placed into test tubes containing cold distilled water. Water was then replaced by the staining solution i.e. 0.1% Commassie blue in 45% methanol and 10% acetic acid and stained overnight. Then gels were rinsed in distilled water and destained with 40% methanol and 10% acetic acid.

E. Determination of molecular weight

(a) By gel filtration on Sephadex G-50 : The molecular weights of native DE-I, DE-II and DE-III from human placenta and fetal liver were determined by gel filtration (284) on a Sephadex G-50 column (1.5 cm x 75 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.5. Proteins of known molecular weight (2 mg each) were used for the calibration of the column. It was operated at a constant flow rate of 10 ml/hour and 1 ml fractions were collected. The void volume (V_o) was estimated as the elution volume of blue dextran and the internal volume (V_i) was determined as the elution volume of di-DNP lysine by measuring the absorbance at 660 nm and 420 nm respectively. For the determination of the molecular weights of DE-I, DE-II and DE-III, purified proteins were loaded on the column and the eluents were monitored for the proteins. From the void volume (V_o), internal volume (V_i) and elution volume (V_e) for each proteins, Kd [$Kd = \frac{V_e - V_o}{V_i - V_o}$] was calculated and a standard curve was obtained by plotting Kd against the log molecular weight of marker proteins and the native molecular weights of DE-I, DE-II and DE-III were determined from this curve.

(b) By SDS-PAGE acrylamide gel electrophoresis : SDS-PAGE under reduced condition was carried out as described earlier for determining the subunit molecular weights. Molecular weight marker proteins were also run in separate tubes and semi-log plot of molecular weight versus R_f (R_f = the distance migrated by the protein/the distance migrated by the tracking dye) was constructed. The molecular weight of the subunits of DE-I, DE-II and DE-III were determined from the standard curve.

F. Heat stability of FABP :

Heat stability of the protein was studied by prior pre-exposing the butanol treated protein preparation at 80^0C for 10 minutes

in a water bath before Sephacryl S-200 gel filtration. After cooling for 1 minute at 0^0C, the protein was incubated with 12-(9-anthroyl) stearic acid at 37^0C for 30 minutes and chromatographed on Sephacryl S-200 column as well as on DEAE cellulose column respectively as described before. Readings were taken at 280 nm for protein and at 364 nm or 12-(9-anthroyl)stearic acid.

G. Effect of different reagents on the binding of 12-(9-anthroyl)stearic acid by FABP :

DE-II and DE-III fractions of FABP, containing 12-(9-anthroyl)stearic acid were lyophilized and dialyzed overnight against cold water. Each of these was then subjected to gel filtration on Sephadex G-50 (1.0 x 25 cm, flow rate 12 ml/hr) equilibrated with (i) 0.01 M Tris-HCl, pH 8.5, (ii) 1 M KCl in 0.01 M Tris-HCl, pH 8.5, (iii) 0.01 M phosphate buffer, (iv) 1 M glycine in 0.01 M Tris-HCl buffer, pH 8.5, (v) 10% ethylene glycol in 0.01 M Tris-HCl buffer, pH 8.5, (vi) 5% Triton X-100 in 0.01 M Tris-HCl buffer, pH 8.5, (vii) 5 mM EDTA in 0.01 Tris-HCl, pH 8.5.

In each case, readings at 280 nm for protein and at 364 nm for 12-(9-anthroyl)stearic acid were taken in a Hitachi Spectrophotometer (Model No. 200-20).

H. Isoelectric focusing (IEF) :

Analytical IEF on polyacrylamide gel was performed by modified method of Wrighley (285) using 5% gel columns. Carrier ampholines of pH 3-10 were used at a final concentration of 20% (v/v). Electric focusing was carried out at 4^0C using 100 V for the first 1 hour, followed by focusing at 200 V for 4 hours. After electrolysis, the course of the pH gradient in the set of gels were determined by cutting a gel, identical to those to be stained, into 20 equal sections and measuring the pH of 1 ml of water extract of each piece. From the other gels, carrier ampholytes were removed by gently agitating the gels in 5% TCA (10 ml per gel) and changing

the TCA at least 5 times at 1 to 2 hourly intervals. After removal of the carrier ampholytes, the gels were stained by immersing for 1 hour in 0.1% Commassie blue, G-250. Destaining was done by washing in a mixture of 7.5% acetic acid and 5% methanol.

I. Preparation of antibody specific for DE-II fraction of FABP :

For raising antibody, FABP was purified by the methods described earlier. DE-II fraction of FABP was dialyzed extensively against distilled water and lyophilized. The homogeneity of the DE-II preparations was checked by PAGE under non-denaturing and denaturing conditions.

Rabbits were immunized with two subcutaneous injections of homogeneous preparation of DE-II in 0.9% saline emulsified in CFA (1:1), given at an interval of 10 days (0.5 mg of DE-II/injection), followed by one injection with IFA. Monomeric DE-II was found to be very weakly immunogenic and thus it was polymerized using EDAC before mixing with either CFA or IFA. The immune sera were collected about 10 days after the last injection and were tested by Ouchterlony double immuno-diffusion test.

J. Immunodiffusion :

Immunodiffusion was carried out essentially as described by Ouchterlony (286) on 1% agarose in 20 mM sodium phosphate buffer, pH 7.5. One central and four/five peripheral wells (0.5 cm in diameter) were punched in the agarose bed (distance between peripheral and central wells was approximately 1.5 cm). Antiserum was applied in the central well and protein samples were applied in the peripheral wells. The diffusion was allowed to take place at 4^{0}C in a moist chamber. Precipitation bands usually appear within 36-40 hours after the application of the antigen.

2. Assay of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP-1-oxidoreductase, G6PD, EC 1.1.1.49)

A. Preparation of subcellular fractions :

The cross-sectional pieces of human placental and fetal liver tissues were taken, chopped and washed with ice-cold distilled water to make them free from blood as far as practicable. The tissues were homogenized in a Potter-Elvehjem glass homogenizer at 0-4^{0}C in 0.25 M sucrose to make a 20% (w/v) tissue homogenate. The cellular components of both human placenta and fetal liver were separated according to the method of Hogeboom (287) with slight modifications (93) which are as follows :

Step I : The homogenate after straining was centrifuged at 800 x g for 10 minutes in an International Refrigerated Centrifuge, K-24. The pellet (P_1) was washed three times with the sucrose solution. The washed pellet was finally suspended in ice-cold triethanolamine buffer, pH 7.5 and was used as the nuclear fraction.

Step II : The combined supernatants from step I were again spun at 10,000 x g for 30 minutes. The pellet (P_2) thus obtained after washing thrice was suspended in the same buffer and was served as the source of mitochondria.

Step III : The combined post mitochondrial supernatants were next centrifuged at 20,000 x g for 30 minutes. The pellet (P_3) was washed thrice with sucrose solution and suspended in ice-cold buffer and was used as the lysosomal fraction.

Step IV : The post lysosomal supernatants were combined and again spun at 1,05,000 x g for 1 hour in a Beckman Ultracentrifuge, Model L3-50. The pellet (P_4) now obtained, suspended in ice-cold buffer, was regarded as the microsomal fraction. The centrifugate, on the other hand, after bringing to the desired concentration

by dilution with ice-cold buffer, was used as soluble supernatant or cytosolic fraction. All steps in the sub-cellular fractionation were carried out at 0-4 ^{o}C.

B. Enzyme assay :

The enzyme was assayed in a medium containing 0.05 M triethanolamine buffer, pH 7.5, 0.13 mM G6P, 0.1 mM NADP, 0.25 µg/ml of enzyme in a total volume of 3 ml. G6PD activity was measured according to the method of Lohr and Waller (288) following the increase in absorbance at 340 nm due to the formation of NADPH.

C. Inhibition of G6PD :

Inhibition of G6PD activity by PAL-CoA or oleic acid was measured in a medium containing 0.05 M triethanolamine buffer, pH 7.5, 0.13 mM G6P, 0.1 mM NADP, 0.25 µg/ml of enzyme and different concentrations of PAL-CoA or oleic acid in a total volume of 3.0 ml. G6PD activity was measured as mentioned above.

D. Effect of FABP on G6PD activity :

Assay mixture contained 0.05 M triethanolamine buffer, pH 7.5, 0.13 mM G6P, 0.1 mM NADP, 0.25 µg/ml enzyme and PAL-CoA or oleic acid in different concentrations in a total volume of 3.0 ml. Concentrations of DE-I, DE-II and DE-III were 25 µg/ml each in case of placenta and 40 µg/ml each in case of fetal liver. In other experiments different concentrations of DE-II and DE-III were used. Enzyme was assayed as indicated above.

3. Estimation of protein

Protein was estimated according to the method of Lowry et al. (289) using BSA as the standard.

4. Statistical analysis

Data were treated statistically using the Student's 't' test (2ꝏ). The variability of the data was presented as mean ± SEM. Differences at $P<0.05$ were considered to be significant.