[Chem. Pharm. Bull. 32(2) 708—715 (1984)]

Carrier Proteins in Human Fetal Serum:[1] Bilirubin-Binding Abilities of Albumin, α-Fetoprotein and Ligandin

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(Received June 3, 1983)

The bilirubin-binding abilities of human serum albumin, α-fetoprotein and ligandin were investigated by employing absorbance spectral measurement, peroxidation and fluorescence measurement techniques. The binding constants of the proteins from adult serum and cord serum were very similar. However, those of α-fetoprotein were slightly smaller than those of albumin. Ligandin had two cooperative binding sites for bilirubin, and the binding constants were of the same order as those of the weaker binding sites of albumin. A study of the effect of linolic acid revealed that the bilirubin bound to α-fetoprotein was more easily liberated in the presence of linolic acid than that bound to albumin binding. The drug-binding abilities of these proteins were also examined, and no significant difference was found between adult serum and cord serum albumins. However, α-fetoprotein appeared not to exhibit drug-binding ability when the peroxidation method was employed. The biological role of α-fetoprotein in fetal plasma may be similar to that of albumin.

Keywords——human serum albumin; α-fetoprotein; ligandin; bilirubin binding; absorbance spectrum; peroxidation method; fluorescence spectra method; difference spectrum method

Catabolism of hemoprotein produces bilirubin, and the efficient removal of bilirubin, particularly in newborn infants, is essential for protection from bilirubin toxicity. Unbound bilirubin destroys many vital cell functions[2,3] and is probably the most critical parameter in establishing the risk factor for bilirubin encephalopathy.[4,5] Bilirubin is transported in the plasma in conjugation with albumin.[6] There is an abundance of albumin in plasma and it has a high binding ability with bilirubin.[7] Moreover, albumin also binds many substances such as dyes, drugs and hormones.[8] These substances are called bilirubin displacers or competitors, and they competitively dissociate bound bilirubin from albumin, allowing bilirubin to pass into the tissue.

α-Fetoprotein (AFP), a fetal glycoprotein, is the subject of considerable attention because of its reappearance in patients with hepatocellular carcinoma, teratocarcinoma,[9] yolk sac tumor and developmental disorders.[10] AFP shares many similarities with albumin, including sequence homology,[11] immunological cross-reactivity[12] and physiological properties.[13] The biological role of AFP remains to be elucidated, but the proposed functions of AFP include the binding of some substances[14–16] and immunoregulatory effects.[17,18] Berde *et al.*[15,19] have recently employed spectroscopic and fluorescence techniques to determine the binding and distance interrelations of fatty acids and bilirubin bound to human albumin and AFP. They have demonstrated that albumin has one strong and two weak bilirubin-binding sites and that AFP has one strong and one weak binding site. The facts that AFP is similar to albumin and that it has binding ability with bilirubin raise the possibility that AFP is a carrier protein of certain substances in fetal tissue. However, the concentration of albumin is larger than that of AFP during the fetal period and albumin has a higher affinity for bilirubin than AFP.[15] Therefore, it may be worthwhile to consider whether AFP plays a more important

role than albumin in bilirubin transport in fetal plasma, if bilirubin displacers such as fatty acids, *etc.* are absent.

Ligandin is another bilirubin-binding protein that is abundant in hepatocytes.[20] It overcomes the mitochondrial respiratory inhibitory effects caused by bilirubin. In addition, the relationship between bilirubin-binding and glutathione *S*-transferase activity was investigated,[21] but the binding ability of ligandin to bilirubin was found to be of the same order as that of the weaker binding sites of albumin.

In the present work, we attempted to clarify the interrelationship of albumin, AFP and ligandin as regards bilirubin-binding ability, with particular attention to the effect of ligands. Despite the fact that there have been numerous reports concerning adult serum albumin, the role of cord serum albumin as a carrier protein remains to be elucidated, although several physicochemical and immunological investigations have been performed. We therefore also compared cord serum albumin with adult serum albumin and investigated the function of both as carriers. Furthermore, the probable role of AFP in fetal tissue is discussed.

Materials and Methods

Materials——Human serum albumin was purchased from Miles Laboratories, Sephadex G-150, Blue Sepharose CL-4B and carboxymethyl (CM)-cellulose were from Pharmacia Fine Chemicals, and peroxidase (Type I) was from Sigma Co. Ampholyte (pH 3.5—10.0) was from LKB Producter AB, and charcoal (Norit A) was from Nakarai Chemical Industries. Other reagents were of analytical reagent grade.

Purification of Albumin, AFP and Ligandin——Albumins were purified from adult serum and cord serum, by employing Blue Sepharose CL-4B, DE-32 and Sephadex G-150. AFP was purified from human cord serum by immunoadsorption and Sephadex G-150, as described previously.[22] Ligandin was purified from human liver by CM-cellulose and glutathione affinity column chromatography.[23] Each protein was homogeneous on disc and SDS polyacrylamide gel electrophoresis.

Delipidation of Purified Albumin, AFP and Ligandin——Albumin and AFP were delipidated by the method of Chen,[24]: briefly, by treatment with charcoal (0.1 mg/ml of protein), adjustment to pH 3.0, incubation at 4 °C for 2 h, neutralization and filtration. Ligandin was exhaustively dialyzed against the buffer and was not further purified.

Adsorption Spectral Shifts of Bilirubin in the Presence of Albumins, AFP and Ligandin——The absorbances of bilirubin and protein bound bilirubins were measured with a spectrophotometer. Bilirubin solutions were freshly prepared by dissolving bilirubin in 0.1 N NaOH and diluting this solution as described with 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, unless otherwise stated. The concentration of bilirubin solution was calculated from the absorbance at 440 nm based on a molar absorptivity of $47500\,M^{-1}\,cm^{-1}$. All operations involving bilirubin solutions were carried out with protection from light.

Peroxidation Method——The binding of bilirubin to the primary binding sites on proteins was determined by using a Hitachi model 200-10 spectrophotometer according to the method of Jacobsen and Wennburg.[25] Two ml of the sample solution and various amounts of bilirubin were prepared in cuvettes and the increase in the absorbance at 440 nm was recorded. The concentration of unbound bilirubin was calculated from the initial oxidation velocity. The initial velocity was determined at various molar ratios of bilirubin to protein. One μM bilirubin was equal to 0.05 OD/min in the present method. These procedures were performed at 25 °C.

Fluorescence Spectral Method——Fluorescence spectra were recorded with a Hitachi 650-50 fluorospectrometer. Excitation of tryptophanyl residues was performed at 294 nm and emission was determined either by scanning from 300 to 400 nm or by measurement at 340 nm. The slit width was 2 nm for excitation and 4 nm for emission. Unless otherwise stated, all measurements were made at room temperature and in 20 mM potassium phosphate buffer (pH 7.4). The binding constants of protein for ligands were determined according to the method of Levine.[26] The quench curve was used to calculate the concentrations of bilirubin that are protein-bound and unbound. The binding of bilirubin to protein was analyzed according to the Scatchard Eq. (1)[27]:

$$\bar{V} = \sum_{i=1}^{n} [(L)K_i/\{(L)K_i+1\}] \qquad (1)$$

where K_i refers to the binding of the ligand to the i-th binding site. The above equation involves the assumption that the binding sites are independent.

Difference Spectrum Method——The difference spectrum was recorded with sample and reference cuvettes using the Hitachi 200-10 spectrophotometer. Bilirubin solution in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and an aliquot of linolic acid at various concentrations in methanol were added to 2 ml of sample solution in the same

buffer. After the mixture had been kept at 37 °C for 20 min, the difference spectrum was recorded against the reference at 25 °C.

Competitive Binding of Bilirubin and Drugs——The competitive binding of bilirubin to protein was studied according to the method of Broderson.[28] Determination of the initial oxidation rate as a function of drug concentration served as a basis for assessment of the displacing effect of the drug. When the drug concentration is large compared to that of albumin, the following Eq. (2) is valid:

$$V/V_0 = K_D D + 1 \quad (2)$$

where V and V_0 are the initial velocities with and without the addition of drug, and K_D and D denoted the drug binding constant to the primary bilirubin binding site on albumin and the drug concentration, respectively. The procedure was as follows; the drug to be tested was added to 2 ml of protein bilirubin solution (bilirubin-to-protein ratio = 0.5) in 10 mM phosphate buffer containing 0.15 M NaCl and 5 mM hydrogen peroxide. The sample solution was incubated at 37 °C for 20 min, then 10 μl of peroxidase solution was added and the concentration of unbound bilirubin was calculated from the initial oxidation velocity. The drug did not influence the rate of enzymic oxidation.

The Effect of Linolic Acid on Bilirubin Bound to Protein——The effect of free linolic acid on bilirubin bound to protein was measured according to the method of Starinsky and Shafrir[29] with a minor modification.[2] Two ml of protein bilirubin solution in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and various concentrations of linolic acid was measured by the peroxidation method.

The Glutathione *S*-Transferase Activity of Ligandin——The glutathione *S*-transferase activity of ligandin was measured with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione (GSH) as substrates, according to the method of Habig *et al.*[30] The ligandin used in the study had a specific activity of approximately 20 units/mg.

Results

Interaction of Albumin, AFP and Ligandin with Bilirubin

Spectral shifts of the bilirubin absorption in the presence of protein were indicative of protein bilirubin interaction. Each protein caused an enhancement of bilirubin absorbance but the behavior of the spectral shift was slightly different in each case (Fig. 1). Albumin shifted the absorption maximum from 440 nm (specific for bilirubin) towards 445 nm, while AFP changed it to 435 nm and ligandin to 445 nm.

Primary Binding Sites of Bilirubin to Albumin, AFP and Ligandin

The binding of bilirubin to the primary binding site was determined by the peroxidation method. In the present paper, the secondary bilirubin binding sites were not investigated by the peroxidation method because of lack of reliability in determining secondary binding constants by measuring the initial oxidation velocity. Scatchard plots of the initial rate data showed a single primary binding site for bilirubin on each of adult serum albumin ($K = 2.2 \times 10^8\ M^{-1}$), cord serum albumin ($K = 2.0 \times 10^8\ M^{-1}$) and AFP ($K = 0.96 \times 10^8\ M^{-1}$), but in the case of ligandin, the addition of bilirubin did not alter the initial rate of peroxidation.[21]

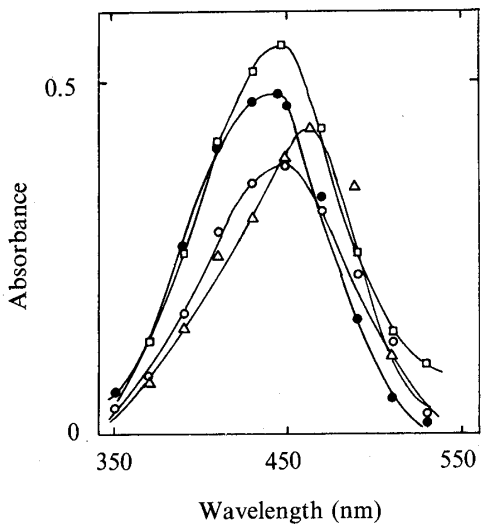

Fig. 1. Spectral Shift of the Adsorption Maximum of Bilirubin (—○—) in the Presence of Human Serum Albumin (—△—), α-Fetoprotein (—●—) and Ligandin (—□—)

The protein concentration in each case was 0.5 μM and the molar ratio of bilirubin to protein was 1.0. The spectral shift changes were measured in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and at 25 °C.

Primary and Secondary Binding of Bilirubin to Albumin, AFP and Ligandin

The binding of bilirubin to the primary and secondary binding sites was determined by measuring the ability of bilirubin to quench the intrinsic protein fluorescence. The quenching of remaining fluorescence by the addition of bilirubin could be analyzed by assuming that the quenching followed a Langmuir adsorption isotherm. The results are shown in Fig. 2 and Table I. However, the Scatchard plots of the binding of ligandin did not follow the simple isotherm. The data were also analyzed by using Hill plots, which indicated cooperative binding of bilirubin to ligandin. The Hill coefficient was $n \doteq 1.5$ and the composite dissociation constant, K', was 1.7 μM. Thus, ligandin has two interacting binding sites.[21]

The Effect of Linolic Acid on the Binding of Bilirubin to Albumin, AFP and Ligandin

The difference spectra produced as a result of bilirubin binding to the proteins are shown in Fig. 3. The spectral changes at 440 nm were different from each other.

The displacement of protein bound bilirubin by linolic acid was also measured by the peroxidation method[28] according to the procedure of Starinsky and Shafrir.[29] The amount

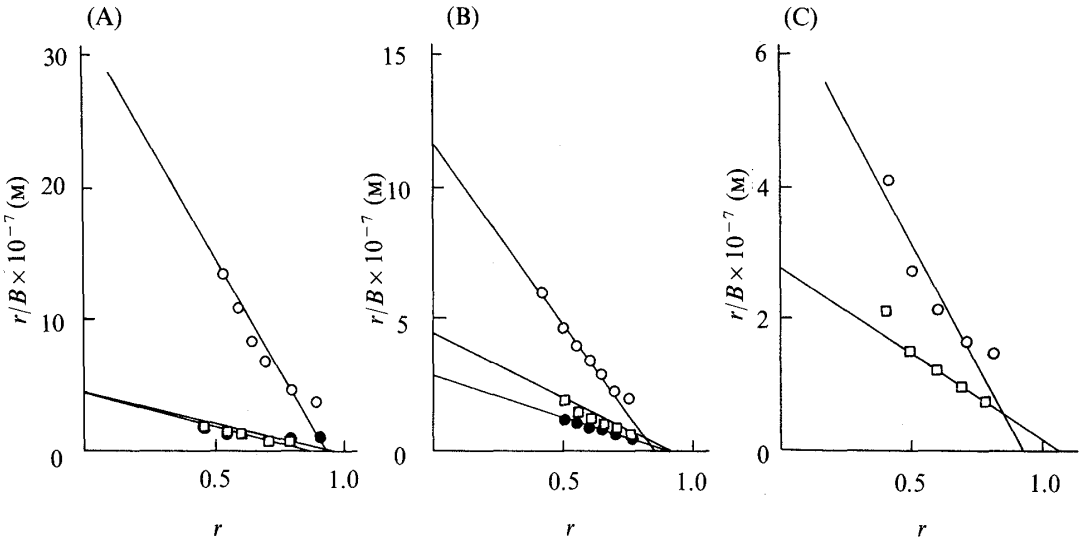

Fig. 2. Scatchard Plots for Titration of Albumin and α-Fetoprotein with Bilirubin

The protein concentrations were 5 μM for human serum (A) and cord serum albumin (B) and 0.5 μM for AFP (C). The fluorescence was measured in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C.
K_1: —○—; K_2: —□—; K_3: —●—.

TABLE I. Association Constants for Bilirubin with Human Albumin or α-Fetoprotein Determined by Fluorescence Quenching

	K_D (M^{-1})		
	HSA	HCA	AFP
K_1	3.1×10^7	1.5×10^7	7.8×10^6
K_2	5.9×10^6	5.7×10^6	5.0×10^6
K_3	5.4×10^6	3.4×10^6	—

The protein concentrations used were 5 μM for human serum (HSA) and cord serum albumin (HCA) and 0.5 μM for AFP. Binding of bilirubin to HSA, HCA and AFP was determined in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C by fluorescence quenching measurements. Binding constants were determined by nonlinear least-squares fitting to the coefficients of the Scatchard equation.

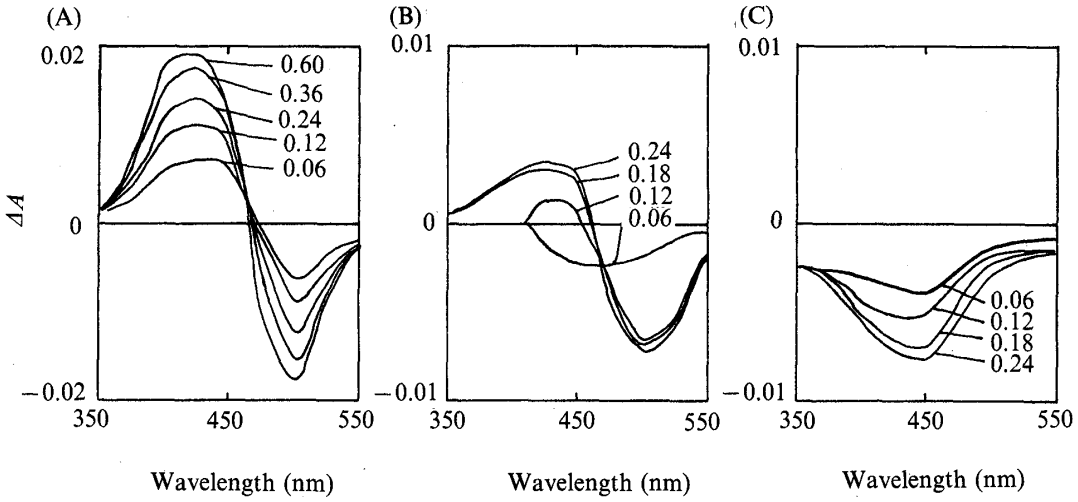

Fig. 3. The Effect of Linoleate on the Bilirubin Binding to Human Serum Albumin, α-Fetoprotein and Ligandin

The protein concentration was 0.5 μM and the molar ratio of bilirubin to protein was 1.0 in each case. The difference spectra were measured in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at 25 °C. The numbers indicate the molar ratio of linoleate to (A) human serum albumin, (B) α-fetoprotein, (C) ligandin.

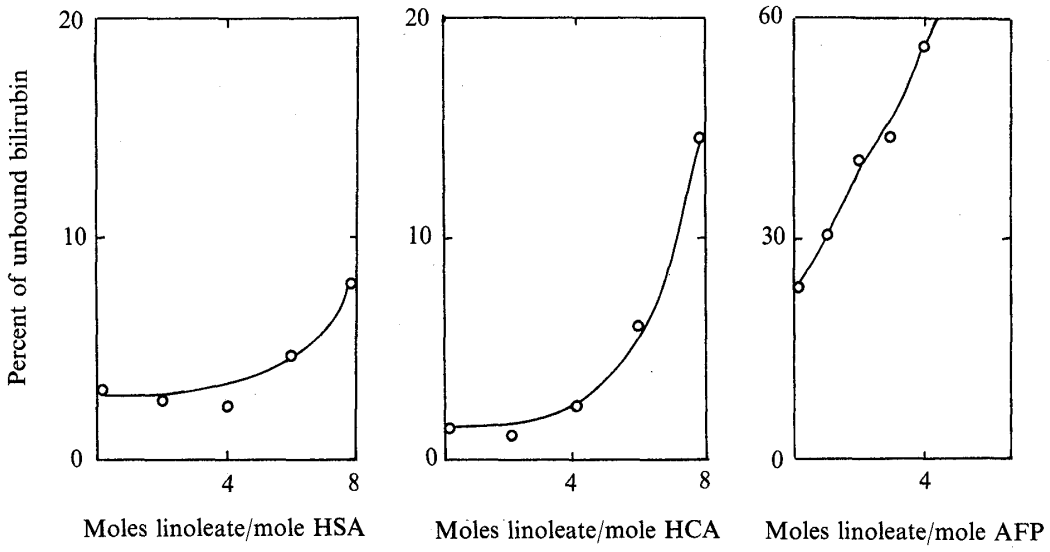

Fig. 4. The Effect of Linoleate/Protein Molar Ratio on the Dissociation of Bilirubin from Proteins

The protein concentrations were 5 μM for human serum and cord serum albumin and 0.5 μM for α-fetoprotein. The experiments were conducted in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at 25 °C. The curves represent the percentages of unbound bilirubin in the mixtures with a constant bilirubin/protein molar ratio of 0.5, to which increasing amounts of linoleate had been added.

of dissociated bilirubin which was oxidized by peroxidase was expressed as a percentage of total bilirubin present in the assay system, and plotted as a function of molar ratio of linolic acid to protein. The curves indicated that below a linolic acid/albumin molar ratio of 3, virtually no free bilirubin was liberated. At the molar ratio of 4 and above there was a definite increase in the oxidation rate of free bilirubin. However, the effect of linolic acid on the bilirubin bound to AFP was distinct. When the linolic acid/AFP molar ratio was increased from 1, the liberated bilirubin increased linearly (Fig. 4). Therefore, the liberation of bilirubin bound to AFP occurred more easily in the presence of linolic acid than that of bilirubin bound

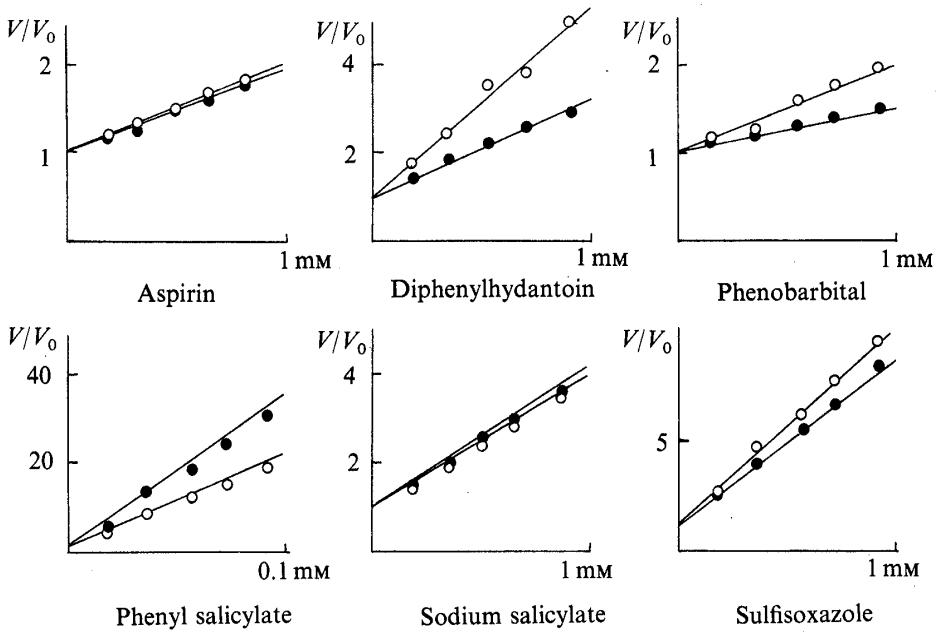

Fig. 5. Free Bilirubin Concentration Relative to the Value without a Displacer (Measured as V/V_0) as a Function of Displacing Drug Concentration

The protein concentrations were 5 μM for human serum (—○—) and cord serum albumin (—●—). The molar ratio of bilirubin to protein was 0.5. The experiment was done in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at 25 °C by the peroxidation method. The slope of the line is equal to the association constant of the drug at the high-affinity site for bilirubin.

TABLE II. Binding Constants of Drugs to the High Affinity Bilirubin Binding Site of Human Serum and Cord Serum Albumins

Drug	K_D ($\times 10^3$) (M^{-1})	
	HSA	HCA
Aspirin	1.0	0.94
Diphenylhydantoin	4.3	2.2
Phenobarbital	0.99	0.50
Phenyl salicylate	21	34
Sodium salicylate	3.0	3.2
Sulfisoxazole	9.2	7.6

The protein concentration was 5 μM and the bilirubin/protein molar ratio was 0.5. The drug was added and the mixture was incubated in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 1% ethanol at 25 °C according to the peroxidation method.

to albumin.

Effect of Various Drugs on the Dissociation of Bilirubin

The competitive binding (with respect to bilirubin) of drugs to albumin was investigated. The results are shown in Fig. 5 and Table II. No significant difference was apparent between the binding abilities of drugs to adult serum and cord serum albumins. The drug-binding to AFP was also investigated, but no binding was detected by the peroxidation method.

Inhibition of the Glutathione *S*-Transferase Activity of Ligandin by Bilirubin

The K_m value of glutathione *S*-transferase activity of ligandin towards 1-chloro-2,4-

dinitrobenzene was 1.25 mM. The inhibition by bilirubin was of competitive type, with K_i = 8.3 μM.

Discussion

In human plasma, many substances bind to albumin and are transported into human tissue. In particular, bilirubin is detoxified when bound to albumin, and is transferred to the liver, where it is decomposed and excreted. AFP has also been proposed to be a carrier protein in fetal plasma, but its biological role is not yet known. We attempted to clarify the relationship between bilirubin and possible carrier proteins.

In the study of the absorption spectral shifts of bilirubin when bound to albumin, AFP and ligandin, slight differences were seen in each case and these may reflect differences in the microenvironments of the bilirubin binding sites. Next, the binding ability of bilirubin with these proteins was investigated by employing peroxidation and fluorescence techniques. The binding constants obtained were consistent with those in other reports.[15,19,25] As regards adult serum and cord serum albumins, there was virtually no difference. The binding constants of AFP to bilirubin were smaller than those of the albumins. Ligandin had two interacting binding sites with binding constants comparable to those of the secondary binding sites of albumin.[21] The effect of linolic acid on the bilirubin bound to protein was examined. Adult serum and cord serum albumins similarly liberated bound bilirubin in the presence of increasing amounts of linolic acid.[29] However, in the case of AFP, the liberated bilirubin was increased linearly and the bilirubin-binding ability of AFP appeared to be more markedly affected in the presence of linolic acid than did that of albumin. In the difference spectral study (Fig. 3), the change in each case revealed the conformational change of bilirubin bound to protein by linolic acid and did not result from the liberation of bilirubin from protein. The binding constants of drugs were also investigated, and both adult serum and cord serum albumins showed similar binding constants. However, AFP did not appear to bind drugs as determined by the peroxidation method. Drug binding was not examined in the case of ligandin.

Since adult serum and cord serum albumins were functionally indistinguishable and were present in large amounts, it is likely that cord serum albumin is responsible for bilirubin transport during the fetal period. AFP was rather different from albumin in that the liberation of bilirubin bound to AFP was more easily accelerated by increasing the amount of fatty acids than it was in the case of albumin, and that no binding ability of drugs to AFP was detected by the peroxidation method. AFP may have a different role in bilirubin transport in hepatocytes,[20] but in the present experiment, it showed weaker bilirubin binding ability *in vitro*. Only trace amounts of ligandin are present in plasma, and ligandin in plasma may simply represent elimination from tissue.

AFP is the first α-globulin developed in fetal plasma; it increases until the first trimester, and thereafter decreases, and the serum level of AFP is of the same order as that in adults within a month after birth. On the other hand, albumin increases from the second trimester and reaches the adult level in the newborn. Drug metabolism during the first trimester is conducted through fetoplacental circulation in the maternal body, because the detoxication system in the fetal body is not developed and AFP can not effectively bind and transport drugs. However, in later stages, albumin is produced and carries these drugs to the detoxication system in the fetal body. In addition, after switch-over from AFP to albumin, albumin has a binding capacity for fatty acids while retaining its bilirubin-binding capacity, and is considered to be a necessary defence system for protection against hyperbilirubinea in the newborn. Therefore, taking into account the sequence homology between AFP and albumin, the hypothesis arises that albumin replaces AFP as a carrier protein in

the human body, as fetal development progresses.

In conclusion, we have investigated the effect of ligands on the bilirubin binding capacities of albumin, AFP and ligandin. These proteins are all effective carriers, but the binding sites of these proteins are rather different. The present and previous results[17,18,31] support the view that AFP plays an important role in the fetal body. Also, taking into consideration the report that ligandin retains but albumin loses bilirubin-binding capacity in the presence of liver cytosol,[32] ligandin may play an important role in bilirubin transport in hepatocytes rather than in plasma. Further investigations is necessary for the clarification of the physical functions of these proteins *in vivo*.

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