Vitamin A transport in plasma of the non-mammalian vertebrates: isolation and partial characterization of piscine retinol-binding protein[1]

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Abstract Studies were conducted to explore vitamin A transport in the non-mammalian vertebrates, especially Pisces, Amphibia, and Reptilia, and to isolate and partially characterize piscine retinol-binding protein. Retinol-containing proteins in fresh plasma obtained from bullfrogs and a turtle exhibited similar properties to those found in mammalian and chicken plasma: i.e., molecular weight of about 60,000–80,000 as estimated by gel filtration and binding affinity to prealbumin on human prealbumin–Sepharose affinity chromatography. In sharp contrast, vitamin A-containing proteins in plasma from larvae of bullfrogs as well as three fishes (carp, blue sharks, and young yellowtails) appeared to be present in plasma as monomeric retinol-binding proteins without any affinity to human prealbumin. On the other hand, plasma vitamin A in the lamprey (Cyclostomes) was found to exist exclusively as an ester form in association with the lipoproteins of hydrated density less than 1.21 g/ml. Piscine retinol-binding protein was isolated from pooled plasma of young yellowtails and was converted (1000-fold purification) to a homogeneous component by a procedural sequence that included gel filtration on Sephadex G-100, chromatography on SP–Sephadex, gel isoelectric focusing, and, finally, polyacrylamide gel electrophoresis. Purified piscine retinol-binding protein showed physicochemical properties distinctly different from the mammalian and chicken retinol-binding proteins examined, i.e., a smaller molecular weight of approximately 16,000, a lower isoelectric point of 4.3, a prealbumin mobility on analytical polyacrylamide gel electrophoresis, and a lack of binding affinity for human prealbumin; however, it displayed similar characteristics in two ways: a 1:1 molar complex with retinol, and a high content of tryptophan (four residues). These results strongly suggest that the piscine retinol-binding protein is a prototype of the specific vitamin A-transporting protein in plasma of the vertebrates, being modified later in evolution, during phylogenetic development of the vertebrates, to acquire a binding site for prealbumin on the molecule.

Supplementary key words retinol-binding protein–prealbumin complex · 3-dehydroretinol · young yellowtail · frog · tadpole · lamprey

It is well established that retinol (vitamin A) is transported in plasma by a specific protein, retinol-binding protein (RBP), which has been isolated and characterized in many mammalian species including man (1, 2), rat (3, 4), monkey (5), pig (6), ox (7), dog (8, 9), and rabbit (10). RBP from all of these species has a molecular weight of close to 20,000, has a single binding site for one molecule of retinol, and migrates in the α-region on electrophoresis. Under physiological conditions, RBP circulates in plasma as a 1:1 molar complex with prealbumin (PA), which has a molecular weight of about 55,000 (11, 12). The association of RBP with PA increases the stability of the retinol–RBP complex (13, 14) and is assumed to prevent the ready filtration of RBP through the renal glomerulus.

On the other hand, very little is known about vitamin A transport in plasma of the non-mammalian vertebrates, with the exception of the finding of an RBP similar to that of mammals in chicken plasma

Abbreviations: RBP, retinol-binding protein; PA, prealbumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

[1] Portions of this work were presented at the Annual Meetings of the Symposium on Chemical Physiology and Pathology, Osaka, November 1974, Japanese Conference on the Biochemistry of Lipids, Kurashiki, June 1975, and Japanese Society of Vitaminology, Sendai, April 1976, and at the 10th International Congress of Nutrition, Kyoto, August 1975.

(15, 16) and egg yolk (17). We now report the finding of distinctly different transport systems for plasma vitamin A in Pisces and Cyclostomes, and also describe the isolation and partial characterization of piscine RBP.

EXPERIMENTAL PROCEDURE

Collection of plasma or serum specimens

Whole blood was drawn from a healthy male human subject, Wistar-strain rats (*Rattus norvegicus*), and a rabbit (*Oryctolagus cuniculus*). A snapping turtle (*Trionyx sinensis japonicus*) was purchased from Nozawa-ya, Tsukiji, Tokyo, and its blood was collected into a heparinized container by decapitation. Blood of two bullfrogs (*Rana catesbeiana*), purchased from Nozawa-ya in August, and of 21 tadpoles (*Rana catesbeiana*) at the premetamorphic stage, collected in Sawara City, Chiba-ken, was kindly drawn by Dr. H. Nagano from the aorta by a nonheparinized syringe as described previously (18).

Blood was also obtained from three fishes: carp (*Cyprinus carpio*), blue sharks (*Pronace glauca*), and young yellowtails (*Seriola quinqueradiata*). Five carp, cultivated for 2 yr, were first anesthetized in water containing MS 222 (Sankyo Pharmaceutical Co., Tokyo), and then whole blood was drawn through the Cuvierian duct by a heparinized syringe. Blue sharks were caught by students of Mr. T. Ishibashi (Misaki Marine School, Yokosuka City) in May 1973 in the Pacific Ocean (Long. 178° E. and Lat. 12° N.), and from each shark 200 ml of blood was collected into a plastic bottle, wrapped with aluminum foil, in which 2 ml each of 10% sodium azide (E. Merck, Darmstadt) and a heparin solution (Sigma, Grade 1; 500,000 units dissolved in 50 ml of distilled water) were added in advance. After standing in a refrigerator overnight, the whole container was deep-frozen and sent to this laboratory, where the frozen upper layer, containing mainly plasma, was recovered by cutting the bottle in half to avoid contamination by the red cells as much as possible.

Two blood collections were made from young yellowtails. One collection consisted of 100 ml of blood from four fish cultivated for 2 yr (a generous gift of Dr. J. Tanaka, Arasaki Branch, Tokai-Regional Fisheries Research Laboratory, Yokosuka City); the blood was collected into a heparinized beaker by means of a caudal fin section. The other collection consisted of 500 ml of blood from 31 young yellowtails that was obtained through the Cuvierian duct by a syringe containing Hepacalin solution (Eisai Pharmaceutical Co., Tokyo). These fish, which were cultivated for 20 months, were kindly supplied by Mr. H. Osuga (Numazu Branch, Shizuoka Prefectural Fisheries Research Laboratory, Numazu City). The former (100 ml) blood sample was used for analytical studies, and the latter was particularly used for the isolation of piscine RBP. Thirteen lampreys (*Entosphenus japonicus*), the generous gift of Mr. S. Kaji (Yatsume Seiyaku, Asakusa, Tokyo), were collected in January 1975 from the Ishikari River, Hokkaido Island, and their whole blood (about 17 ml) was collected in a heparin-containing tube by gravity after open incision of the heart.

Each plasma or serum sample was separated from erythrocytes by centrifugation at 2,000 *g* for 20 min. A portion of the fresh plasma or serum was immediately used for gel filtration on Sephadex G-200 and for vitamin A determination. The remainder was kept frozen in the dark at −20°C until needed.

Column chromatography

The molecular weight of plasma retinol-containing protein in several species was estimated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals AB, Uppsala). Blue dextran polymer with a molecular weight of 2×10^6 (Pharmacia) was added to the plasma prior to gel filtration. For isolation of piscine RBP, Sephadex G-100 and SP-Sephadex C-50 (Pharmacia) were prepared as described in previous reports (3, 10, 16). Affinity chromatography on a column of human PA–coupled Sepharose was performed by the method of Vahlquist, Nilsson, and Peterson (19). Human PA was purchased from Boehringwerke AG, Germany and further purified by preparative PAGE before use (3, 12). Thirty mg of human PA was covalently coupled with 4.5 g of CNBr-activated Sepharose 4B (Pharmacia), and the coupling efficiency was found to be 96%. The sample was applied to the column, and the unbound protein (no affinity to human PA) was washed out by 0.05 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. Protein-bound retinol was then eluted with distilled, deionized water adjusted to pH 10 by adding NH_4OH as the dissociating solution. Each column chromatographic procedure was carried out in a cold room at 4–5°C. Specific details of typical illustrative examples are given in the legends to the appropriate figures (Figs. 1–4).

In order to concentrate a large quantity of the RBP-containing fraction during the isolation procedure, we initially tried ultrafiltration with use of a 20/32-in seamless cellulose tubing as previously reported (10, 16, 20). However, the recovery of piscine RBP was extremely poor (<10%). Hence, we finally adopted a hollow fiber desalting–concentration sys-

tem, Amicon model DC2 equipped with a Diaflo cartridge, model H1 P8 (Amicon Corp., Lexington, MA); recovery of piscine RBP was found to be about 90%. To concentrate the RBP-containing pools in a small quantity, a standard stirred cell, Amicon model 52 with a Diaflo membrane UM 2 (Amicon), was usually employed with a good yield.

Gel electrophoresis

Disc polyacrylamide gel electrophoresis (PAGE) in 0.4 M Tris–glycine buffer, pH 8.9, was conducted in a cold room according to the method of Davis (21). Prior to protein staining, the gel was examined for fluorescence specific for protein-bound retinol under a UV lamp with a wavelength of 365 nm. The electrophoretic profile of proteins stained with Amido black 10B (Tokyo Chemical Industry Co., Tokyo) was recorded densitometrically with use of Densitron model Pan-J (Jookoo Sangyo Co., Tokyo) employing a filter of 570 nm. Sodium dodecyl sulfate (SDS)-containing PAGE was carried out according to the method of Weber and Osborn (22), particularly to determine the molecular weight of purified piscine RBP simultaneously with that of chicken RBP. The SDS (99% pure, SPS-4) was purchased from Nakarai Chemicals, Ltd., Kyoto. The chicken RBP used was isolated by the following sequence of procedures as previously reported (16) with modifications. *1*) Chromatography on DEAE–Sephadex, pH 7.0, with a linear gradient of 0.1–0.6 M NaCl; *2*) chromatography on DEAE–Sephadex, pH 7.2, with a linear gradient of 0.25–0.45 M NaCl; *3*) gel filtration on Sephadex G-100; *4*) chromatography on a human PA affinity column; and *5*) semipreparative PAGE. The other proteins of known molecular weight used as standards for the analysis were obtained from Schwarz-Mann, Orangeburg, NY.

Analytical and preparative isoelectric focusing in polyacrylamide gel was carried out according to the method of Righetti and Drysdale (23) with specific details as previously described (24). Carrier ampholytes (Ampholine, LKB Produkter AB, Stockholm) in the pH range 2.5–4, and 4–6 were used. Each run was allowed to continue for 17–23 hr at a constant voltage of 400 V, at 0°C, using an apparatus (Medical Research Apparatus Corp., Boston) with a cooling system (Neslab Instruments, Inc., Portmouth, NH.). After focusing, each gel was sectioned by Yeda macrotome (Yeda Scientific Instruments, Rehovot, Israel) into 17–27 segments, and each section was dispersed in 1.0 ml of distilled water. The pH of each eluate was measured at room temperature and protein-bound retinol was assayed by measuring the relative intensity of specific fluorescence. Specific details are indicated in the legend to Fig. 5. When needed, the proteins were also stained after focusing with 0.1% Amido black 10B for 10 min, after prewashing with 5% trichloroacetic acid overnight (25).

Amino acid analysis

Because of the very limited amount of pure piscine RBP available, analyses were carried out on only one sample (0.35 mg) subjected to acid hydrolysis for 24 hr. Both human and rabbit RBP (holo-RBP; H2), as described in a previous report (24), were simultaneously subjected to acid hydrolysis under the same conditions. The samples were hydrolyzed in 4 N methanesulfonic acid (Eastman Kodak Co., Rochester, NY) containing 0.2% 3-(2-aminoethyl)indole (Eastman) in evacuated sealed tubes at 110°C, as described elsewhere (10, 12, 26). Amino acid analyses were carried out on a JEOL amino acid autoanalyzer, model JLC-5AH (JEOL, Akishima City, Tokyo).

In vitro binding of radioactive retinol with piscine RBP and its displacement by various vitamin A derivatives

[*Carbinol*-^{14}C]retinol (10 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England. All-*trans* retinol (Sigma Chemical Co., St. Louis, MO), and all-*trans* 3-dehydroretinol and retinoic acid (the generous gifts of Hoffmann-LaRoche, Basel) were used for displacement experiments as competitors. Purified piscine RBP, 50 pmol dissolved in 200 μl of 0.02 M potassium phosphate buffer, pH 7.4, containing 0.2 M NaCl was added to 5 μl of ethanol solution of radioactive retinol (1000 pmol; 2.2×10^4 dpm). Each incubation with or without a 170-fold molar excess of competitor was done for 14 hr in a cold room at 4–5°C, and then the sample was subjected to gel isoelectric focusing as described above. After focusing, the amount of protein-bound [^{14}C]retinol in each sliced gel was assayed by a Packard Tri-Carb liquid scintillation spectrometer, model 3380 (Packard Instruments, Downers Grove, IL), after digesting each gel segment with 3% Protosol (New England Nuclear, Boston, MA) in a toluene–Omnifluor (New England Nuclear) scintillator at 60°C overnight.

Study on quantitative binding affinity of piscine RBP for human PA

To evaluate the lack of specific binding site of piscine RBP for human PA, displacement of human RBP previously bound to human PA–Sepharose was quantitatively measured by adding a molar excess of piscine RBP. Human RBP (85 μg in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.4, and 0.5 M NaCl) was added

to the human PA–Sepharose (138 μg of human PA in 0.1 ml of the gel) as described earlier. Incubation was carried out 5 min at room temperature, and then the Sepharose was separated from the supernatant by centrifugation at 1000 rpm for 5 min. After several washings with the same buffer, a maximum of 36.5 μg of human RBP was found to bind with the Sepharose as judged by single radial immunodiffusion according to the method of Mancini, Carbonara, and Heremans (27). The washed human RBP-containing human PA–Sepharose was then employed for competitive binding analysis with piscine, chicken, rabbit RBP, and bovine serum albumin (see Fig. 9).

Other procedures

Absorbance and absorption spectra were measured with a Shimadzu double beam spectrophotometer, model UV-200. Fluorescence measurements were carried out with a Hitachi fluorescence spectrophotometer, model 204. Fractions eluted from columns were assayed for protein-bound retinol by measuring the relative intensity of fluorescence with excitation at 330 nm and emission at 460 nm. All fluorescence measurements were conducted at 22°C, and the optical density of each sample was never allowed to exceed 0.5 at the excitation wavelength.

The efficiency of energy transfer (T) was calculated by the following equation (28).

$$T = \frac{ODa}{ODd} \cdot \frac{Fd}{Fa}$$

where ODa and ODd are the optical densities of the solution at 330 nm and 280 nm, Fd is the fluorescence intensity at 460 nm by excitation at 280 nm and Fa is that at 460 nm by excitation at 340 nm, respectively.

Protein concentrations were estimated from the absorbances at 280 nm, and by the method of Lowry et al. (29), with bovine serum albumin as a standard. The extinction coefficient ($E^{1\%}_{1cm}$) of RBP at 280 nm was estimated by measuring the absorbance at 280 nm and the protein concentration by the method of Lowry et al. (29) on the same solution of pure RBP.

Retinol concentrations of whole plasma, and of the RBP-containing pools from the initial fractionation procedures, were determined according to the method of Thompson et al. (30). Ethanol–hexane 1:2 extracts of whole plasma from the young yellowtail and lamprey were analyzed by TLC with silica gel G plates (E. Merck), using a developing solvent system of petroleum ether–ethyl ether–acetic acid 80:20:1. After drying the plates, the spots were detected by fluorescence specific for retinol under a UV lamp. In addition to retinol and retinoic acid, all-*trans* retinyl palmitate (Daiichi Pure Chemicals Co., Ltd., Tokyo) and retinylaldehyde (Sigma) were employed as authentic standards.

Analytical ultracentrifugation was performed according to the method of Havel, Eder, and Bragdon (31) in order to obtain the lipoproteins of the lamprey plasma. Densities of duplicate samples were adjusted to 1.21 g/ml by adding solid KBr (Wako Pure Chemical Industries, Ltd., Osaka), and then centrifugation was continued at 105,000 *g* for 42.5 hr at 15°C using a rotor RP-40 with a Hitachi ultracentrifuge, model 65P.

RESULTS

Retinol-containing proteins in native plasma of various species

Molecular weight estimates on gel filtration (**Fig. 1**). When fresh plasma or serum from the rabbit and bullfrogs were chromatographed on the same column of Sephadex G-200, retinol-containing proteins were eluted in relative retention volumes consistent with a molecular weight of approximately 60,000–80,000. In fresh plasma from the snapping turtle, however, protein-bound retinol was eluted in two peaks, corresponding to proteins of molecular weights of about 75,000 and 20,000, respectively.

In contrast, retinol-containing protein in plasma of tadpoles and three fishes (young yellowtails, carp, and blue sharks) was eluted as a single peak, with an elution volume consistent with a smaller molecular weight, in the range of 20,000.

Human PA affinity chromatography (**Fig. 2**). When human whole serum was applied on a column of human PA–coupled Sepharose, protein-bound retinol was initially adsorbed to the immobilized PA, and then eluted as peak 2 by means of a low ionic strength alkaline eluting solution. As shown in Fig. 2, the retinol-containing proteins in plasma or serum from the rat, chicken, turtle, and frog revealed the same elution profiles, demonstrating their ability to bind to the human PA. In sharp contrast, the retinol-containing protein in plasma of the tadpole and three fishes was immediately eluted as peak 1.

Isoelectric point. When fresh plasma or serum (each 0.3 ml) was subjected to isoelectric focusing on a cylinder (0.45 × 8 cm) of polyacrylamide gel, a single fluorescent band of protein-bound retinol was detected prior to staining. Isoelectric points of retinol-

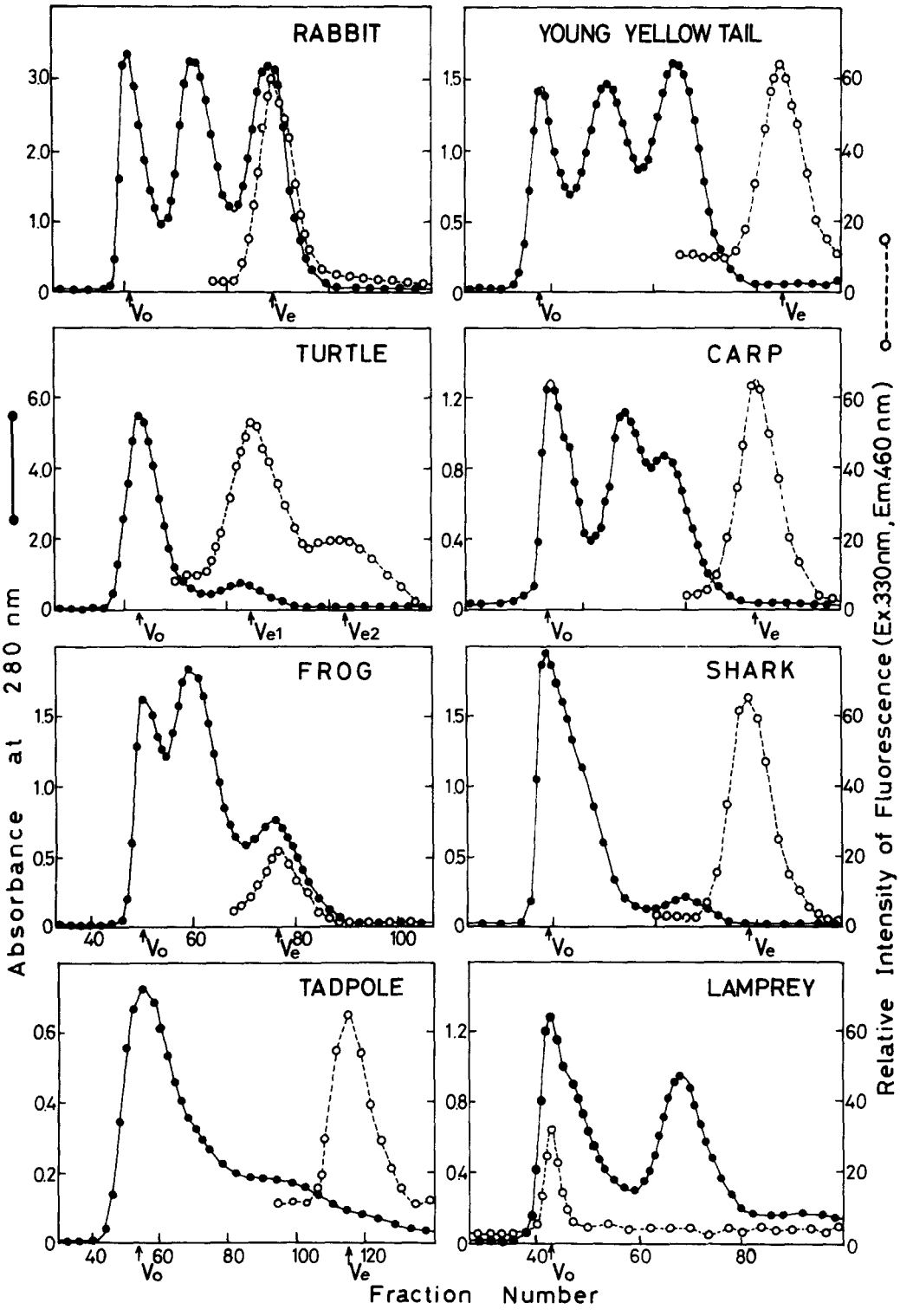

Fig. 1. Molecular weight estimation of the plasma retinol transport system in several vertebrate species. Gel filtration of fresh plasma or serum (each 3 ml, with the exception of tadpole plasma, 0.7 ml) was carried out on a Sephadex G-200 column (2.7 × 80 cm), equilibrated with 0.02 M potassium phosphate buffer, pH 7.4, containing 0.2 M NaCl. Fractions of 4 ml each were collected at a flow rate of 14 ml/hr in a cold room. A thinner column (1.2 × 84 cm) of Sephadex G-200 was used for study on tadpole plasma, and fractions of 0.5 ml each were collected at a flow rate of 10 ml/hr. Each sample applied to the column contained a small amount of blue dextran polymer 2,000 (Pharmacia) in order to determine the void volume (indicated by the small arrows labeled *Vo*). The relative elution volumes (*Ve/Vo*) for the plasma retinol-containing proteins (indicated by arrows labeled *Ve*) in the samples assayed were rabbit 1.67, turtle 1.54 and 1.91, frog 1.56, tadpole 2.00, young yellowtail 2.20, carp 2.10, shark 2.10, and lamprey 1.00. Plasma concentrations of retinol (ng/ml) were also found to be rabbit 700, turtle 167, frog 59, tadpole 169, young yellowtail 775, carp 864, shark 20, and lamprey 98. Measurements of fluorescence specific for retinol in both plasma and fractions eluted from the columns are described under Experimental Procedure.

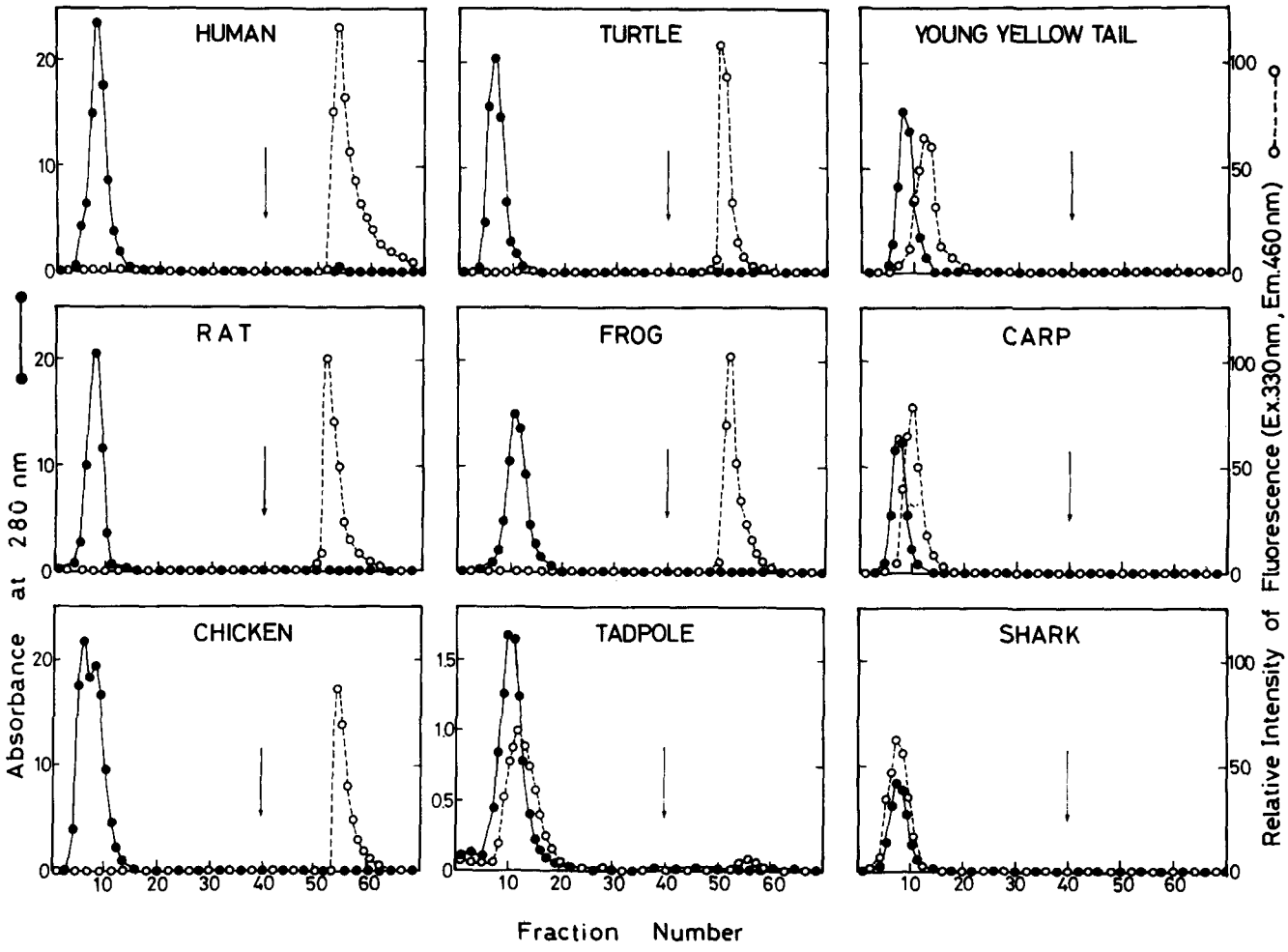

Fig. 2. Elution profiles of plasma retinol-containing proteins in various vertebrate species on human prealbumin (PA) affinity chromatography. For each species, 3 ml of the native plasma was applied to a column (1.2 × 12 cm) of Sepharose 4B, covalently coupled with 30 mg of human PA, and equilibrated with a starting buffer of 0.05 M Tris-HCl, pH 7.4, containing 0.5 M NaCl. In tadpole, the combined fractions containing protein-bound retinol after Sephadex G-200 column chromatography (see Fig. 1) were used after concentration. After the sample was allowed to stand within the column for about 20 min, almost all of the proteins were exhaustively washed out by the starting buffer, and then retinol-containing proteins, bound to the immobilized human PA, were dissociated by means of a low ionic strength alkaline eluting solution (distilled, deionized water, adjusted to pH 10 with concentrated NH_4OH) as indicated by the arrows. Fractions of 2 ml each were collected at a flow rate of 20 ml/hr and assayed for protein (absorbance at 280 nm) and protein-bound retinol (specific fluorescence), respectively.

containing protein in chicken and turtle plasma were found to be 4.9 and 5.1, respectively. On the other hand, retinol-containing proteins in fish plasma exhibited distinctly lower isoelectric points: young yellowtail, 4.3, and carp and blue shark, 4.1.

Vitamin A transport in lamprey plasma. Retinol-containing protein in fresh plasma of the lamprey was eluted in the void volume on Sephadex G-200 chromatography (Fig. 1), suggesting that a macromolecular protein was responsible for vitamin A transport. When the fresh plasma (3 ml) was subjected to ultracentrifugation with the density of 1.21 g/ml for 42.5 hr, most of the vitamin A was recovered in the top fraction of lipoproteins[2] (recoveries of vitamin A were 75.5 and 83.2% in duplicate samples). Moreover, it was observed by TLC analysis that vitamin A in the fresh plasma existed exclusively in an ester form. The ethanol–hexane extract of the lamprey plasma exhibited a single fluorescent spot consistent with retinyl palmitate (R_f 0.88), whereas

[2] The top fraction was found to contain two lipid-staining bands on both analytical PAGE and agarose gel electrophoresis: a major band migrated in a post-albumin region on PAGE (prestained by Sudan Schwarz 4B), and in the α-globulin region on agarose gel electrophoresis (stained by Oil Red O), respectively.

that of young yellowtail plasma showed a single spot of retinol (R_f 0.31).

Isolation procedure of piscine RBP

Purification of retinol-containing protein from plasma of young yellowtails was attempted as a representative piscine RBP in terms of the molecular weight, the very low or no affinity for human PA, and other characteristics as described above. RBP was isolated by the following sequence of procedures: gel filtration on Sephadex G-100, chromatography on SP-Sephadex, preparative isoelectric focusing in polyacrylamide gel, and, finally, semipreparative PAGE. These procedures resulted in a homogeneous RBP with about 1000-fold purification. The yield at each isolation step of RBP is summarized in **Table 1**.

When a portion of the pooled plasma from young yellowtails was subjected to gel filtration on Sephadex G-100, a single protein-bound retinol peak was eluted, being well separated from other major plasma proteins as shown in **Fig. 3**.

The combined fractions of the fluorescent peaks from all three successive runs on the same gel filtration column were further subjected to SP–Sephadex chromatography as indicated in **Fig. 4**. The protein-bound retinol was eluted as a single peak by 0.1 M sodium acetate buffer, pH 6.5. The fluorescent fractions, however, were found to contain more than eight contaminating protein bands on analytical PAGE. Prior to isoelectric focusing, small amounts of vitamin A were added to the combined fractions in order to achieve a better yield by increasing the stability of holo-RBP in electric fields (24). The pooled and concentrated sample (4.5 ml containing 16.2 mg of protein and 80.7 μg of retinol) was added and mixed with 230 μg of retinol dissolved in 50 μl of ethanol, and then dialyzed exhaustively against distilled water.

TABLE 1. Purification steps and yields of piscine RBP

Fraction	Protein	RBP[a]	RBP/protein	Yield
	mg	*mg*		%
Whole plasma	10,870	10.84	0.001	100.0
Sephadex G-100	112	9.35	0.084	86.3
SP–Sephadex C-50	16.2	4.51	0.279	41.6
Isoelectric focusing and PAGE	1.21	1.21[b]	1.000	11.2

[a] Estimated from the concentration of retinol measured by fluorescence based on one binding site for retinol per molecule of RBP.

[b] Retinol was exogenously added to the sample on a 1:1 molar basis prior to purification steps of both isoelectric focusing and PAGE.

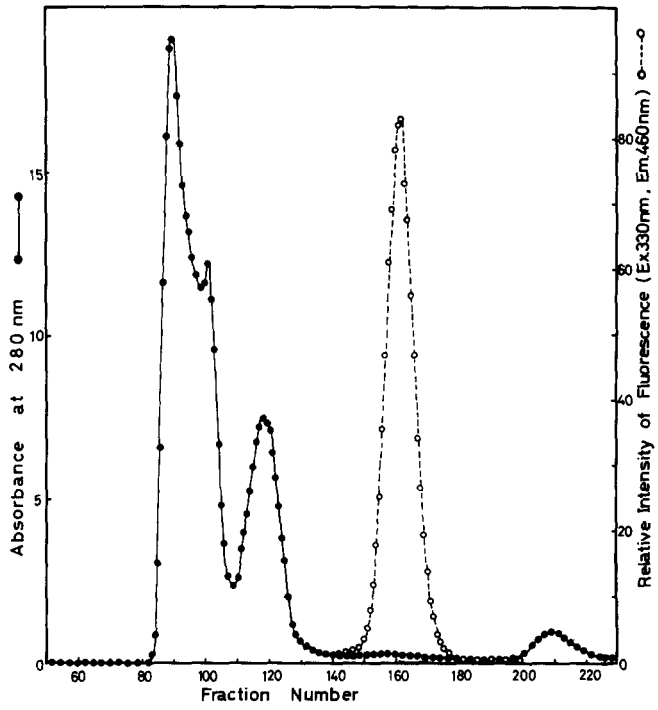

Fig. 3. Gel filtration of piscine (young yellowtail) whole plasma on a column of Sephadex G-100. The pooled fresh plasma (85 ml), containing 3.6 g of protein and 64.6 μg of retinol, was applied to a column (5 × 130 cm) of Sephadex G-100 equilibrated with 0.02 M potassium phosphate buffer, pH 7.4, containing 0.2 M NaCl. Fractions of 15 ml each were collected at a flow rate of 50 ml/hr. The RBP-containing (fluorescent) fractions (147–180) were pooled for further purification.

When a portion of the dialyzed pool was subjected to preparative isoelectric focusing in a polyacrylamide gel, the protein-bound retinol was separated into two peaks; a major one focused at pH 4.3 and a minor one at pH 4.0 (microheterogeneity), respectively (upper panel *A*, **Fig. 5**). The two fluorescent peaks were found to correspond with the respective protein bands as indicated in Fig. 5 (lower panel *B*). Two additional runs of isoelectric focusing were carried out and the major fractions focused at pH 4.3 were combined. The pool, however, was found to be contaminated with ampholytes in large amounts (approximately 10 mg per 1 mg of RBP), which could not be removed simply by means of dialysis against distilled water.

In order to eliminate the contaminating ampholytes, we finally adopted a semipreparative PAGE method as suggested by Vesterberg (32). The lyophilized sample was initially dissolved in 1.5 ml of 10 mM ammonium bicarbonate solution, and then mixed with 0.34 μg of retinol in 10 μl of ethanol prior to dialysis against the same solution. After

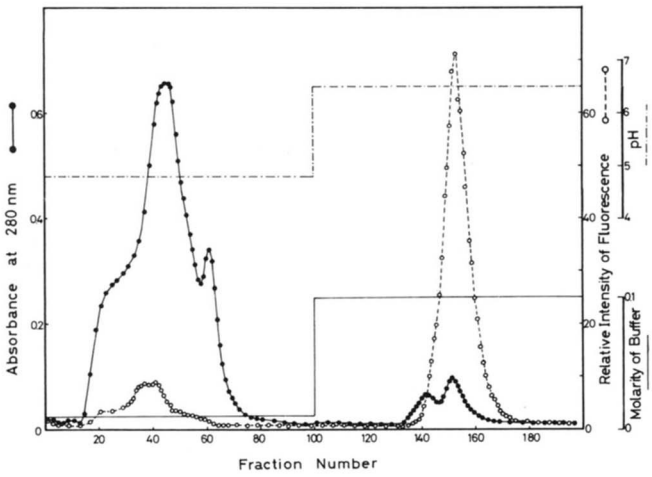

Fig. 4. Chromatography on SP-Sephadex of piscine RBP-containing pool obtained after three runs of the gel filtration on Sephadex G-100 (see Fig. 3). The combined fractions were concentrated by a hollow fiber desalting–concentration system, and dialyzed against 0.01 M sodium acetate buffer, pH 4.8, overnight. The dialyzed portion (68 ml containing 112 mg of protein and 167 μg of retinol), obtained after centrifugation at 15,000 rpm for 10 min to remove insoluble materials, was chromatographed on a column (2.1 × 28 cm) of SP-Sephadex C-50 equilibrated with the same buffer. Chromatography was carried out by a stepwise elution using 200 ml each of 0.01 M sodium acetate buffer, pH 4.8, and 0.1 M sodium acetate buffer, pH 6.5. Fractions of 2 ml each were collected at a flow rate of 16 ml/hr. The RBP-containing fractions (137–170) were pooled for further purification. Some minor fluorescence, a peak of which was eluted around fraction 40, was found to be non-specific for protein-bound retinol.

dialysis overnight, the whole sample was applied to a column (1.2 × 10 cm) of polyacrylamide gel, and electrophoresis was conducted at a constant voltage of 180 V for 4 hr at 0°C. The contaminating Ampholine was found to migrate mostly with the tracking dye (bromophenol blue). Protein-bound retinol was extracted from the minced gel containing specific fluorescence by four changes of distilled water (each 1.5 ml). After re-addition of retinol and successive dialysis against 10 mM NH_4HCO_3, the RBP-containing solution was finally lyophilized. Recovery of RBP in this procedure was 72%. The final preparation of the RBP was used for partial characterization as described in this report.

Partial characterization of piscine RBP

Electrophoretic mobility. When 20 μg of piscine RBP was subjected to analytical PAGE, a homogeneous protein band with fluorescence specific for retinol was observed. The relative mobility of the RBP (R_m 0.63) was distinctly greater than that of bovine serum albumin (R_m 0.54).

Molecular weight. The purified RBP was also found to be a single band by SDS gel electrophoresis. The molecular weight was estimated to be approximately 16,000, distinctly less than that of the chicken RBP examined simultaneously (**Fig. 6**).

Spectral studies. The ultraviolet absorption spectrum of the piscine RBP gave two peaks with maxima at 280 nm and 330 nm, and the absorbance ratio (330 nm/280 nm) was found to be 0.92 (**Fig. 7A**). The extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm was 20.6 for the purified RBP.

Uncorrected fluorescence spectra were recorded under several conditions. When the RBP was excited at 340 nm, a peak of emission was recorded with a maximum at 460 nm. The excitation spectrum for RBP, with emission fixed at 460 nm, showed two peaks with maxima at 280 nm and 330 nm. The emission spectrum displayed two major peaks with maxima at 340 nm and 460 nm when the protein was excited at 280 nm (Fig. 7B). Efficiency of energy transfer from 340 nm (tryptophan residue) to 460

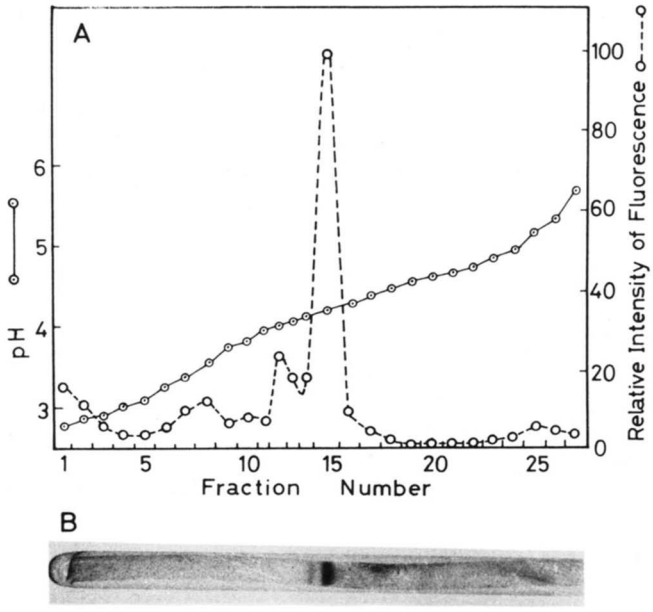

Fig. 5. Isoelectric focusing in preparative (*A*) and in analytical (*B*) polyacrylamide gels of the RBP-containing pool obtained after chromatography on SP-Sephadex (see Fig. 4). After concentration with Diaflo membrane UM 2 and dialysis against distilled water, the sample (4.5 ml containing 16.2 mg of protein) was divided into three portions. Each 1.5 ml was applied to a column (1.2 × 13 cm) of 4% polyacrylamide gel that contained 2% (w/v) ampholytes, pH 2.5–6 (a combination of 1% each of Ampholine of pH 2.5–4 and 4–6). Focusing was carried out at a constant voltage of 400 V, at 0°C for 23 hr, and then the gel was sectioned into 27 pieces with the intervals as indicated in the figure, each piece being dispersed in 1 ml of distilled water overnight. Both protein-bound retinol (○ – – – ○) and pH value (⊙ —— ⊙) were assayed in each eluate. The RBP-containing fraction (number 15) was used for further purification. As shown in the lower panel *B*, a major band, stained by Amido black 10B, was found to be identical with the fluorescent peak of fraction 15 in the upper panel *A*.

nm (retinol) in piscine RBP was calculated to be 24.2%, which was found to be significantly lower than that of the chicken RBP (40.9%) and rabbit RBP (42.0%) examined simultaneously.

Amino acid composition. The result of the amino acid analysis of piscine RBP is presented in **Table 2** and is compared with analyses of human and rabbit RBP. The amino acid composition of piscine RBP was generally similar to that of the other two proteins. Piscine RBP was also found to be fairly rich in aromatic amino acid residues; in particular, there were four residues of tryptophan, as was found with the human and rabbit protein. The major differences, however, were lower levels of valine and phenylalanine, and a higher content of isoleucine in piscine RBP. Other smaller differences were also observed with regard to several other amino acid residues.

Ligand specificity. When piscine RBP was incubated with [^{14}C]retinol, the radioactivity was detected as a single peak on isoelectric focusing, the pI of which was found to be 4.3 (**Fig. 8A**). The radioactive peak was completely displaced by adding a 170-fold molar excess of both retinol (panel *B*) and 3-dehydroretinol (panel *D*), and was markedly reduced by retinoic acid (panel *C*).

Affinity for human PA. When a portion of purified piscine RBP (100 μg) was applied on a human PA affinity column, almost all of protein-bound retinol was recovered as peak 1, as was found with the retinol-containing proteins in native fish plasma (Fig. 2). To study further the lack of specific binding affinity of piscine RBP for human PA, displacement of human RBP previously bound to human PA–Sepharose was

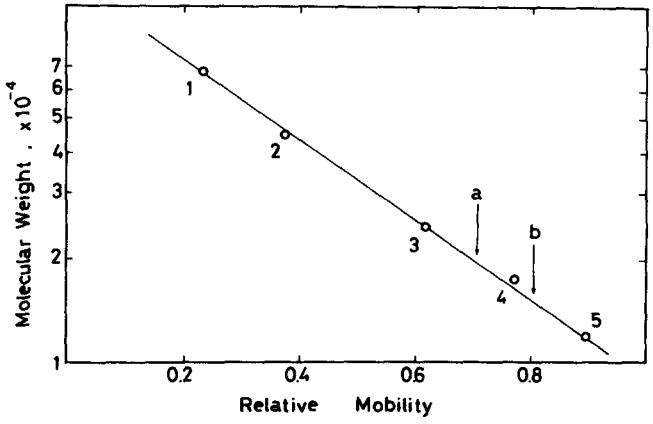

Fig. 6. Estimation of molecular weight of purified piscine RBP on SDS-containing PAGE. The standard proteins used were: *1*, bovine serum albumin; *2*, ovalbumin; *3*, chymotrypsinogen A; *4*, myoglobin; *5*, cytochrome c. The arrows show the observed relative mobilities for purified chicken (*a*) and piscine RBP (*b*), respectively.

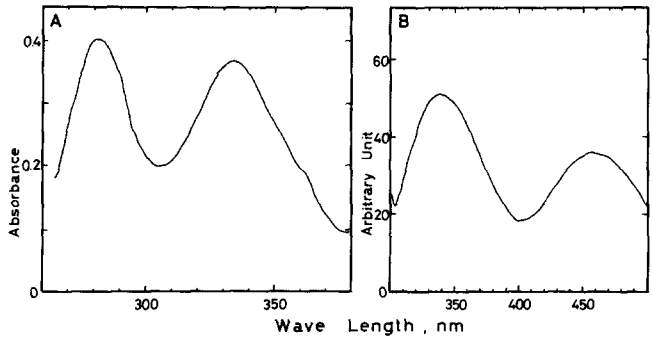

Fig. 7. Ultraviolet absorption spectrum (*A*) and fluorescence emission spectrum with excitation at 280 nm (*B*) of purified piscine RBP.

quantitatively examined by adding a molar excess of the piscine protein, as shown in **Fig. 9**. In sharp contrast to both chicken and rabbit RBP, none of the displacement was observed by even a 10-fold molar excess of piscine RBP similar to bovine serum albumin.

DISCUSSION

The present study has clearly demonstrated a distinct transport system for vitamin A in Pisces, either fresh water fishes (i.e., carp) or marine fishes in-

TABLE 2. Amino acid compositions of piscine, human, and rabbit RBP

	Residues per Molecule[a]			
	Piscine RBP		Human RBP	Rabbit RBP
Amino Acid	Observed	Nearest Integer	Nearest Integer	
Lysine	7.68	8	10	10
Histidine	3.66	4	2	4
Arginine	10.52	11	14	14
Aspartic acid	21.60	21–22	26	30
Threonine	9.02	9	9	9
Serine	7.65	8	10	10
Glutamic acid	14.85	15	18	15
Proline	4.90	5	7	7
Glycine	10.43	10–11	12	10
Alanine	10.45	10–11	14	12
Half-cystine	3.78	4	6	6
Valine	6.30	6	12	13
Methionine	3.77	4	4	4
Isoleucine	4.61	5	4	3
Leucine	12.78	13	13	12
Tyrosine	6.82	7	8	7
Phenylalanine	4.18	4	10	12
Tryptophan	4.11	4	4	4
Total		148–151	183	182

[a] Calculations for residues per molecule of RBP were based on assumed presence of Tyr + Phe, 11 residues in piscine RBP, and Tyr + Phe + Lys + His, 30 residues in human RBP and 33 residues in rabbit RBP.

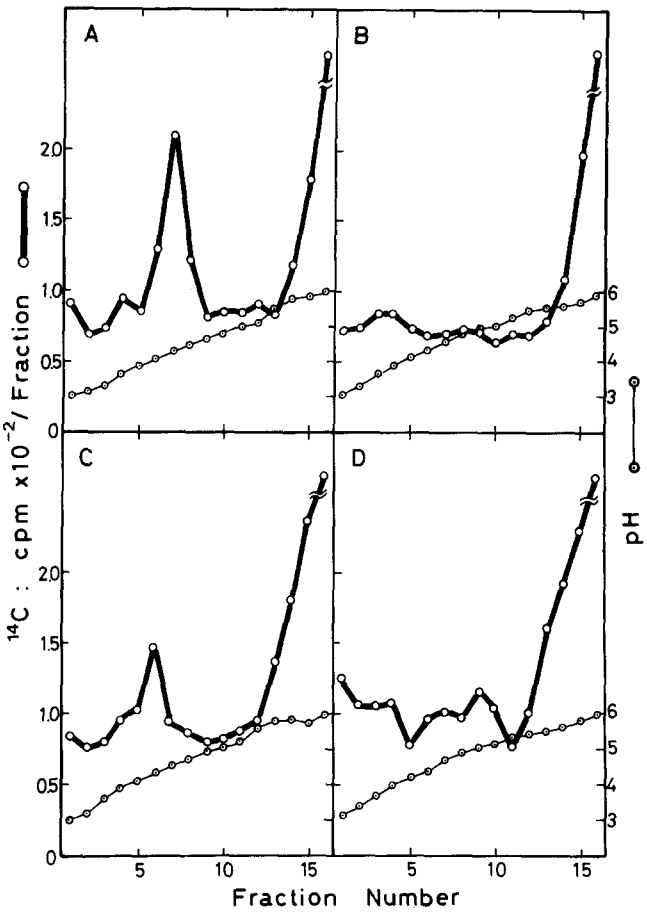

Fig. 8. Ligand specificity of purified piscine RBP. Displacement of [^{14}C]retinol (*A*) bound to the RBP by 170-fold molar excess of competitors, retinol (*B*), retinoic acid (*C*), and 3-dehydroretinol (*D*), respectively. Experimental conditions are described under Experimental Procedure. After focusing, pH value (⊙ —— ⊙) and radioactivity (○ —— ○) in each gel segment were measured.

cluding cartilaginous (i.e., blue sharks) and bony fishes (i.e., young yellowtails). In these species, plasma vitamin A circulates as retinol bound to a specific protein, retinol-binding protein, that circulates as a monomer and not in the form of a complex with prealbumin. In contrast, the vitamin A transport system previously studied in various mammalian species (1–10) and in chicken (15–17) is comprised of RBP that circulates in the form of the RBP–PA complex.

Since no information has been available about vitamin A transport in the non-mammalian vertebrates other than the chicken, it was of interest to examine the molecular characteristics of retinol-containing proteins in native plasma, using both gel filtration on Sephadex G-200 (Fig. 1) and human PA affinity chromatography (Fig. 2). The major protein-bound retinol fractions in the plasma of the turtle and the bullfrog were eluted in relative retention volumes consistent with a molecular size of about 75,000, being similar to those of the rabbit (Fig. 1) and various other mammalian species and of the chicken as previously reported by Muto, Smith, and Goodman (8). Of interest is the observation that the protein-bound retinol in plasma from a snapping turtle was separated into two peaks, suggesting that one was complexed RBP and the other was monomeric RBP similar to those demonstrated in a young dog (8). It was also reported by Heller (7) that bovine RBP circulated exclusively in the uncomplexed form even in fresh plasma, although this finding is inconsistent with a previous report (8). In any event, these retinol-containing proteins were found to be able to bind a human PA, as shown in the turtle (Fig. 2) and as reported in the ox (7), respectively. Despite a high degree of immunological specificity of RBP within a given vertebrate order (8), considerable information is now available demonstrating the binding affinity of PA to heterologous RBP in a number of interspecies combinations: human PA to monkey (5), porcine (6), bovine (7), rabbit (24), and rat RBP (33), and human RBP to chicken PA (17) and rat PA (34), respectively. In addition, it was found that human PA was able to interact effectively with retinol-containing proteins of the turtle and bullfrog as indicated in Fig. 2. In contrast, vitamin A in the plasma of tadpoles (larvae of bullfrogs) as well as three fishes was transported as protein-bound retinol that was eluted on gel filtration as a protein of distinctly

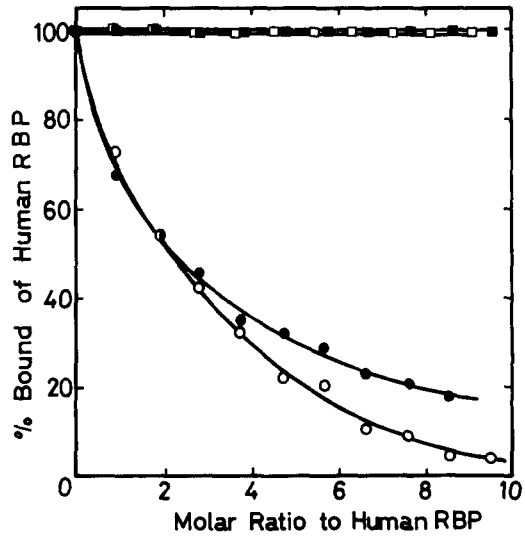

Fig. 9. Lack of specific binding site of piscine RBP for human PA. Displacement of human RBP bound to human PA–Sepharose by in vitro additions of rabbit (● —— ●), chicken (○ —— ○), and piscine RBP (□ —— □), and bovine serum albumin (■ —— ■), respectively. (See Experimental Procedure.)

smaller size (Fig. 1) and did not show any binding to human PA (Fig. 2 and 9). Taken together with these findings, it is now concluded that a similar transport system for plasma vitamin A exists widely in the vertebrates of classes higher than postmetamorphic Amphibia.

The principal aim of this study, however, was to isolate and partially characterize piscine RBP. Despite general difficulty in collecting a large quantity of blood from the fish, pure RBP was successfully obtained from 250 ml of plasma (obtained from 31 young yellowtails cultivated for 20 months). The final preparation with about 1000-fold purification (Table 1) exhibited a single band on analytical PAGE, although a microheterogeneity was observed on isoelectric focusing in gel (Fig. 5), generally similar to the RBP isolated in various other species. Because of the very limited supply, no specific antiserum against the piscine RBP was achieved to examine immunological properties of the pure protein.

Of particular interest is the fact that piscine RBP revealed the following physico-chemical properties, being distinctly different from the RBP as previously reported: a smaller molecular weight of approximately 16,000 on SDS-containing PAGE (Fig. 6), a prealbumin mobility on PAGE consistent with a low isoelectric point of 4.3 (Fig. 5), and lack of a specific binding site for human PA (Fig. 9).

Amino acid analysis of piscine RBP (Table 2) showed significantly less phenylalanine and valine residues and more isoleucine, as compared with human and rabbit RBP determined simultaneously. Hence, it is evident that certain important structural differences must exist among these three proteins, particularly with regard to those aspects of structure involved in the binding site for PA as well as the antigenic determinations[3].

Despite these distinctive properties, piscine RBP was found to be similar to the mammalian and chicken RBP in several ways, including the presence of one binding site on RBP for one molecule of retinol (i.e., the formation of a 1:1 molar retinol–RBP complex), and the presence of a high content of tryptophan (four residues) in the molecule. Spectral studies of piscine RBP showed virtually identical ultraviolet absorption spectra and fluorescence excitation and emission spectra as those of human and chicken RBP. The ratio of the absorbance at 330 nm (protein-bound retinol) to 280 nm (protein itself) and $E_{1cm}^{1\%}$ at 280 nm of piscine RBP were found to be 0.92 and 20.6, respectively, from which a molar ratio of retinol to the RBP was calculated to be 0.77, using a molar extinction coefficient of 46,000 for retinol (35). When piscine RBP was excited at 280 nm, the emission spectrum displayed two peaks with maxima at 340 nm and 460 nm. The first peak presumably represents the emission spectrum of the protein itself (particularly its tryptophan residues), whereas the peak at 460 nm represents that of retinol bound to RBP. This emission spectrum indicates that there is efficient transfer of energy within the holo-protein, presumably mainly from excited tryptophanyl residues to the bound retinol. Efficiency of energy transfer in piscine RBP, however, was found to be much lower than those in both chicken and rabbit holoprotein, suggesting certain structural differences, presumably involving the distances between tryptophan residue and the chromophore.

It was also disclosed that [^{14}C]retinol previously bound to piscine RBP was greatly displaced by a large molar excess of retinoic acid and was completely replaced by all-*trans* 3-dehydroretinol (Fig. 8), which was also able to bind with human RBP as reported by Horwitz and Heller (36). Therefore, the finding strongly suggests that there is a common transport protein (i.e., RBP) for both all-*trans* retinol and 3-dehydroretinol, which dominates in the fresh water fishes.

One of the most interesting findings in this study is that vitamin A in plasma of the lamprey circulates exclusively as an ester form in association with lipoproteins rather than RBP (Fig. 1). The lampreys usually come up in large groups from the sea to the rivers for spawning in autumn, and spawn in the upper streams as their gonads mature during a few months, usually April and May. In Japan the lamprey has often been recommended as a diet efficacious for curing night blindness because of extraordinarily high contents of vitamin A in the flesh. Hence, it is strongly suggested that the peculiar vitamin A transport system in the lamprey found here is related to the unusual tissue distribution of vitamin A ester, about 80% of which is present in the gut and flesh (37), as may well correspond to the hypervitaminosis A in the rat. Vitamin A predominantly exists as an ester in association with plasma lipoproteins, whereas the RBP level markedly decreases, as reported previously (38). However, further data would be needed in order to determine whether or not the lamprey system is representative of all Cyclostomes. Obviously a study of vitamin A transport in plasma will be required, particularly in the

[3] No immunological cross-reactivity was observed when the purified piscine RBP was tested against monospecific antisera of human, rat, rabbit, and chicken RBP, respectively (unpublished observations). The finding supports the conclusion that no displacement occurred when carp serum was tested in radioimmunoassay systems of human and rat RBP as previously reported (8).

hagfishes which are more primitive than the lamprey and entirely marine without metamorphosis.

The evidence now available from this and previous reports indicates that four different transport systems for vitamin A and its derivatives are available in the vertebrates as follows, *1*) retinol–RBP circulating as a tight complex with PA; *2*) monomeric retinol–RBP circulating as such, without high affinity for PA; *3*) retinylester (and carotinoids)–lipoproteins; and *4*) retinoic acid–albumin system (39), respectively. The last two are likely to represent non-specific or collateral systems, which in turn function in a certain abnormal or exceptional state, carrying the vitamins to the peripheral tissues in a manner that is not finely controlled or regulated. It has been suggested that RBP serves the physiological role of specifically delivering vitamin A to appropriate sites in target tissues (38); this delivery process may involve specific receptor sites for RBP on the cell surface in target tissues (40).

An important question arises as to how the renal loss of large amounts of monomeric RBP can be prevented in the fish, in spite of its small molecular weight. Although very little information is available about pore size of the fish glomerulus, it was reported by Bieter (41) that no proteinuria was induced in the aglomerular toadfish, whereas distinct albuminuria with some leakages of exogenous hemoglobin itself was observed in both catfish and eel (the glomerular kidney group) when a solution of homologous hemoglobin (mol wt 64,000) was intravenously injected. Moreover, it is of great interest as reported by Bulger and Trump (42) that the marine teleost has evolved a unique transport system of macromolecules that permits a rapid translocation of intact particles (as well as protein such as egg white lysozyme) from the tubular lumen to the lateral intercellular spaces without direct contact with the lysosomal enzymes. Hence, these findings may well support the contention that a monomeric piscine RBP may be protected in part from glomerular filtration in the aglomerular kidney group, and is also efficiently salvaged as an intact protein (after glomerular filtration) by the tubular reabsorption system, which is specific and predominant in marine teleosts.

In any event, it is concluded from the findings obtained in the present study that piscine RBP is a prototype of the specific vitamin A-transporting proteins found in the vertebrates and was later modified so as to acquire a binding site for prealbumin in the molecule itself during phylogenetic development.

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