Vitamin A transport in chicken plasma: isolation and characterization of retinol-binding protein (RBP), prealbumin (PA), and RBP—PA complex

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Abstract Vitamin A-transporting protein in chicken plasma was purified by column chromatography on DEAE-Sephadex and Sephadex G-100; the protein formed a complex of retinol-binding protein (RBP) with prealbumin (PA). The molecular weight of the 1:1 molar complex was estimated to be 76,000 by gel filtration, and the sedimentation coefficient ($s_{20,w}$) was found to be 5.2 S. RBP and PA were dissociated from the purified complex by means of CM-Sephadex column chromatography. Purified RBP contained 1 mole of vitamin A bound per mole of RBP. The molecular weight of RBP was determined to be 20,000 by gel filtration on Sephadex G-75, 19,000 by SDS–disc gel electrophoresis, and 20,500 by sedimentation equilibrium analysis. The $s_{20,w}$ was calculated to be 2.0 S. The molecular weight of PA was determined to be 56,000 by gel filtration, 52,000 by sedimentation equilibrium analysis, and 13,000 by SDS–disc gel electrophoresis. The $s_{20,w}$ was calculated to be 3.9 S. From these findings it was concluded that PA consists of four subunits, each with a molecular weight of approximately 13,000. Peptide mapping experiments suggested that the subunits were identical. No carbohydrates were detected in either RBP or PA. Chicken RBP and PA were immunologically distinct from those of human and rat.

Supplementary key words molecular weight · amino acid analysis · subunit · peptide mapping · antisera

It is well established that vitamin A is transported bound to a specific plasma protein, retinol-binding protein (RBP), in both man (1, 2) and rat (3). Purified human and rat plasma RBP have a single binding site for one molecule of retinol, α mobility on disc gel electrophoresis, and a molecular weight of approximately 20,000. In both species, RBP forms a tight complex with plasma prealbumin (PA) and normally circulates as a 1:1 molar protein–protein complex with PA (1–5). Despite these similarities, no immunological cross-reactivity between human and rat RBP has been observed (3, 6).

The present study was undertaken to explore whether or not a similar transport system for vitamin A exists in the chicken, a nonmammalian vertebrate. During the course of this study, Mokady and Tal (7) reported the isolation of RBP from chicken plasma and some physicochemical properties, e.g., a molecular weight of about 19,000. On the other hand, Muto, Smith, and Goodman (6) had already observed that the molecular weight of vitamin A-containing protein in fresh chicken plasma is approximately 60,000–80,000, as determined by gel filtration. However, no convincing information is available regarding an entire system of vitamin A transport in chicken plasma. We now describe procedures for the isolation of the RBP–PA complex of chicken plasma and the dissociation into the component proteins, RBP and PA. We also describe in detail the physicochemical properties of the individual proteins. It is also clearly demonstrated that chicken RBP and PA are immunologically distinct and different from the respective proteins in man and rat. Moreover, purified chicken PA appears to be a tetramer of four identical subunits and is thus similar to human and rat PA.

EXPERIMENTAL PROCEDURE

Serum

Blood (4 l) was collected after decapitation of the chickens, and the serum was immediately separated. A small portion of the fresh serum was applied to a Sephadex G-200 column in order to estimate the molecular size of the native form of the vitamin A-transporting protein. The remaining serum (2 l) was frozen immediately after collection and stored until needed.

Portions of this work were presented at the Annual Meeting of the Japanese Biochemical Society in Tokyo in October 1972.

Abbreviations: RBP, retinol-binding protein; PA, prealbumin; SDS, sodium dodecyl sulfate.

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Column chromatography

Column chromatography using DEAE-Sephadex A-50 (Pharmacia) was conducted as described in previous publications (1, 3). CM-Sephadex C-50 (Pharmacia) was equilibrated with 0.05 M acetate buffer, pH 5.4. Specific details are given in the appropriate figure legends. Column chromatography was carried out in a cold room at 4–5°C.

The molecular weights of purified proteins were estimated by gel filtration on standardized columns of Sephadex G-75, G-100, and G-200 according to the method of Whitaker (8).

Electrophoresis

Polyacrylamide disc gel electrophoresis in 0.4 M Tris–glycine buffer, pH 8.9, was performed according to the method of Davis (9). The separating gel was prepared with 7% acrylamide, usually without the concentrating gel. For electrophoresis, a constant current of 3 mA per tube was employed. After electrophoresis, the gels were examined for fluorescence of protein-bound retinol under ultraviolet light. The proteins were then stained with 1% amidoschwarz 10B in 7% acetic acid for 3 hr and destained with 7% acetic acid. The molecular weights of purified proteins were estimated by SDS–disc gel electrophoresis as described by Weber and Osborn (10). The relative mobilities of different proteins were plotted against the logarithms of their molecular weights. The standard proteins of known molecular weight were obtained from Mann Research Laboratories except for the following: RNase A was purchased from Nutritional Biochemical Corp., DNase from Seikagaku Kogyo Inc. (Tokyo), bovine pancreatic trypsin from Sigma Chemical Co., and insulin and glucagon from Novo Industrial Corp. (Denmark).

Urea–polyacrylamide gel disc electrophoresis was carried out according to the method of Möller and Chrambach (11). The gel solution contained 8 M urea and 7% acrylamide in 0.02 M formate–KOH buffer, pH 3.0.

Analytical ultracentrifugation

Sedimentation velocity studies were carried out with a Hitachi model UCA-1 ultracentrifuge equipped with phase-plate schlieren optics. All experiments were carried out using 4 mg/ml purified protein in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. Centrifugation speeds of 60,000 rpm and 55,430 rpm were used for purified RBP, PA, and RBP–PA complex at 20°C. Calculations of sedimentation coefficients were carried out according to Schachman (12). Partial specific volumes for the calculations were estimated from the amino acid analyses (13).

Sedimentation equilibrium was performed by the technique of Yphantis (14). Specific details are given in the legends to Fig. 7.

Amino acid analysis

Protein samples (0.1 mg) were hydrolyzed in 5.7 N HCl at 108°C for 24, 48, and 72 hr. Amino acid analyses were carried out on an automated amino acid analyzer (JEOL model JLC-6AH). Cysteine was estimated by amino acid analysis after performic acid oxidation (15). Tryptophan was analyzed by the method of Edelhoch (16).

Peptide mapping

Each 100 μg of RBP, PA, or RBP–PA complex was dissolved in 85 μg of distilled water and boiled for 10 min. Each sample was then digested in 0.1 M NH_4HCO_3 (pH 8.5) containing 5% (w/w) tosylphenylalanyl chloromethanetrypsin (Worthington) for 22 hr at 37°C.

Peptide mappings of trypsin hydrolysates were performed on cellulose thin-layer plates (precoated, abrasion-resistant; 20 × 20 cm, 0.1 mm thick; E. Merck). 100 μg of each hydrolysate (10 μl) was applied to the plate. Ascending development was performed in *n*-butanol–acetic acid–water 4:1:2 (v/v) at 23°C for 12 hr. Chromatography in the second dimension was performed in *n*-butanol–acetic acid–pyridine–water 5:1:4:4 (v/v) at 23°C for 10 hr (17). The plate was air dried and sprayed with cadmium–ninhydrin and phenanthrenequinone (18, 19).

Preparation of antiserum, and immunological methods

Antiserum against purified RBP or PA was prepared in rabbits. Animals were immunized by intracutaneous injections of a solution of 0.5 mg of pure RBP or PA emulsified in 1 ml of complete Freund's adjuvant. 2 wk later, the rabbits received a booster injection of the same antigen. Blood was collected 3 wk later. The antiserum was assayed for specificity and titer against pure RBP or PA by immunodiffusion using the method of Ouchterlony (20) and by immunoelectrophoresis according to the micromethod of Scheidegger (21).

Other procedures

Absorption spectra were usually measured with a Hitachi Perkin-Elmer model 139 spectrophotometer.

Fluorescence measurements were made with a Hitachi model 204 spectrophotofluorometer. Fractions eluted from columns were assayed for protein-bound retinol by measuring the relative intensity of fluorescence at 470 nm using an excitation wavelength of 330 nm. In the figures, the values for relative intensity of fluorescence refer to the values obtained with excitation and emission at these wavelengths. More highly purified protein fractions were also assayed for protein-bound retinol by measuring absorbance at 330 nm. Fluorescence spectra of purified proteins were recorded automatically with a Shimadzu model RF 502 spectrophotofluorometer at 25°C.

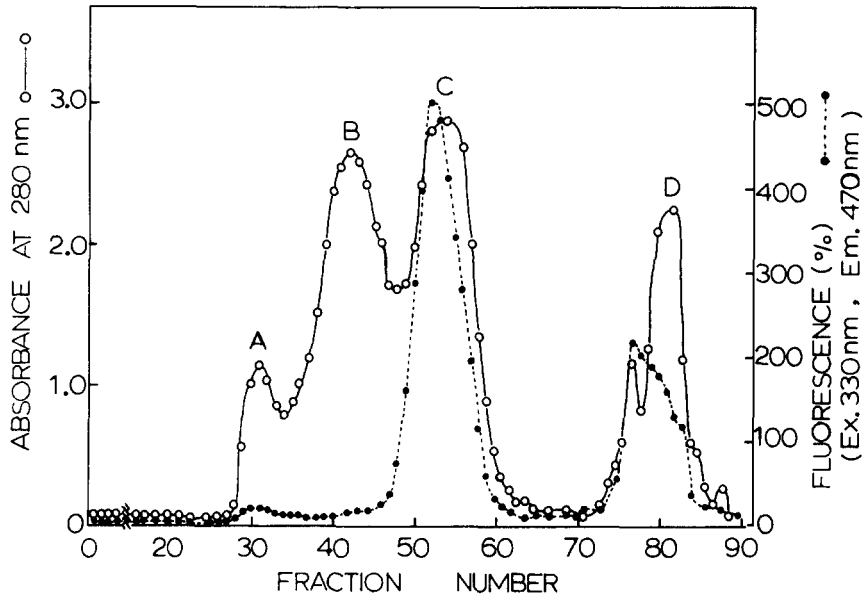

Fig. 1. Gel filtration of chicken whole serum on a column of Sephadex G-200. Fresh chicken serum (4.5 ml) was applied to a column (2 × 53 cm) of Sephadex G-200 equilibrated with 0.02 M potassium phosphate buffer, pH 7.5, containing 0.2 M NaCl. Fractions of 2.5 ml were collected at a flow rate of 15 ml/hr. The void volume of the column (V_0) was at fraction 31. The fractions were assayed by fluorescence with excitation and emission maxima at 330 and 470 nm, respectively. The center of the peak of protein-bound retinol was in fraction 52 ($V_e/V_0 = 1.70$). In contrast, the peak of fluorescence that eluted around fraction 80 did not represent retinol and did not show peak maxima at these wavelengths.

Protein concentrations were estimated by measuring absorbance at 280 nm and by the method of Lowry et al. (22) with bovine serum albumin as a standard. The extinction coefficients ($E^{1\%}_{1cm}$) of purified RBP, PA, and RBP–PA complex at 280 nm and protein concentration by the method of Lowry et al. (22) were determined on the same solution of purified protein. Direct measurement of the retinol level in chicken serum was performed by the trifluoroacetic acid method as modified by Roels and Mahadevan (23).

The neutral carbohydrates in proteins were determined by the orcinol method (24), and amino sugars were determined with an amino acid analyzer.

Protein solutions obtained from several purification steps were concentrated by using VisKing dialysis tubing (20/32) as an ultrafiltration membrane (25). Recovery of protein-bound retinol was found to be more than 85%.

RESULTS

Properties of RBP from whole serum

When pooled chicken whole serum, containing 550 ng of retinol/ml, was subjected to gel filtration on Sephadex G-200, protein-bound retinol was obtained as a single peak ($V_e/V_0 = 1.70$) that eluted slightly before albumin (peak C), as shown in **Fig. 1.** As indicated below, the apparent molecular size (70,000–80,000) represents that of the chicken RBP–PA complex. The fluorescence shown in peak D was not found to be specific for retinol and was later eliminated by dialysis of whole serum prior to gel filtration.

Purification of the RBP–PA complex

Several attempts were made to obtain a completely pure RBP–PA complex, and the following sequence of procedures was finally adopted. Specific details of chromatographic procedures are given in the individual figure legends.

First DEAE-Sephadex column chromatography. Proteins from 2 l of chicken serum were separated into five peaks (peaks E–I) by DEAE-Sephadex column chromatography (**Fig. 2**). Major fluorescence, being specific for protein-bound retinol, was eluted in peak H. On the other hand, minor fluorescence that was eluted in albumin (peak G) was found to be uncharacteristic for retinol with regard to its excitation and emission maxima. The eluates comprising the fluorescent (blue-green) peak H were then pooled and concentrated by ultrafiltration for further purification.

Second DEAE-Sephadex column chromatography. Proteins in peak H (above) were further separated by DEAE-Sephadex chromatography into four peaks (peaks J–M) by a stepwise elution as indicated in **Fig. 3.** Protein-bound retinol was eluted as a single peak (peak M), and most of the contaminating proteins were effectively eliminated by this procedure. Gel filtration on a Sephadex

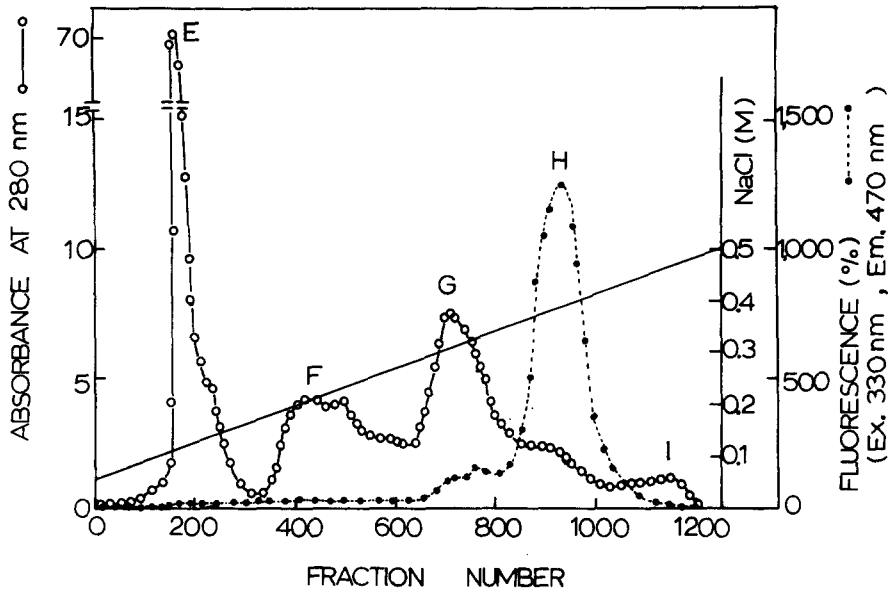

Fig. 2. Chromatography on DEAE-Sephadex of chicken whole serum. Chicken whole serum (2 l) was applied to a column (10.5 × 50 cm) of DEAE-Sephadex equilibrated with 0.02 M potassium phosphate buffer, pH 7.5, containing 0.05 M NaCl. Elution was carried out with a linear gradient of NaCl from 0.05 to 0.5 M. Fractions of 20 ml were collected at a flow rate of 400 ml/hr. The RBP-containing (fluorescent) fractions were pooled (peak H, fractions 820–1020) for further purification. The very small peak of fluorescence shown with peak G (fractions 650–800) did not represent retinol.

G-200 column revealed that protein-bound retinol in peak M had a molecular size similar to that observed in whole serum.

Third DEAE-Sephadex column chromatography. Proteins in the RBP pool of peak M obtained from the preceding column chromatography were further separated

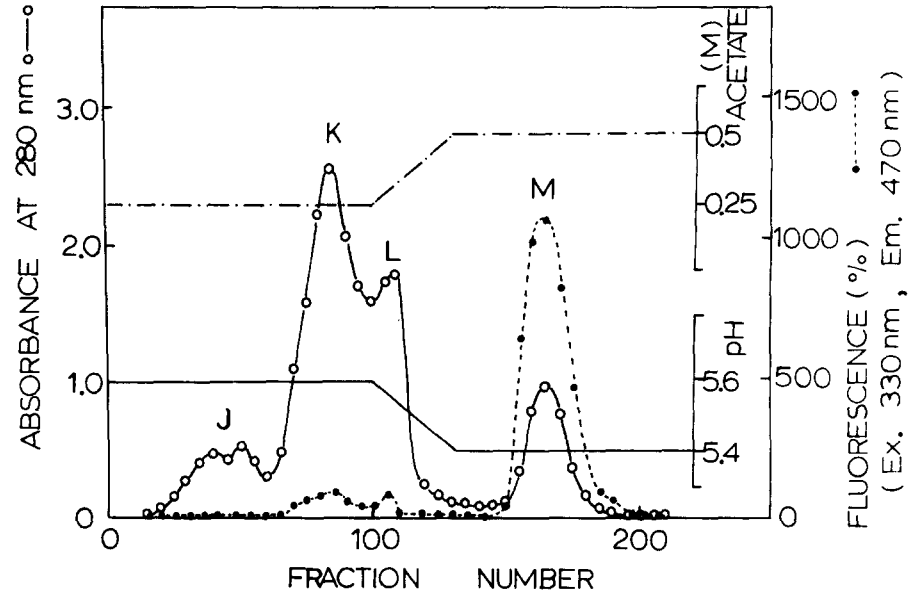

Fig. 3. The second DEAE-Sephadex column chromatography of a portion of the RBP pool (peak H) obtained after the first DEAE-Sephadex chromatography (see Fig. 2). The sample (250 ml, containing 9.8 g of protein) was dialyzed against 0.25 M acetate buffer, pH 5.6, centrifuged at 10,000 *g* for 30 min to remove insoluble material, and applied to a column (5 × 30 cm) of DEAE-Sephadex equilibrated with the same buffer. Elution was carried out by a stepwise elution using 1 l of 0.25 M acetate buffer, pH 5.6, and 1 l of 0.5 M acetate buffer, pH 5.4, respectively. Fractions of 10 ml were collected at a flow rate of 25 ml/hr. The RBP-containing fractions were obtained as peak M (fractions 150–190). The very minor fluorescent peaks shown with peaks K and L did not represent retinol.

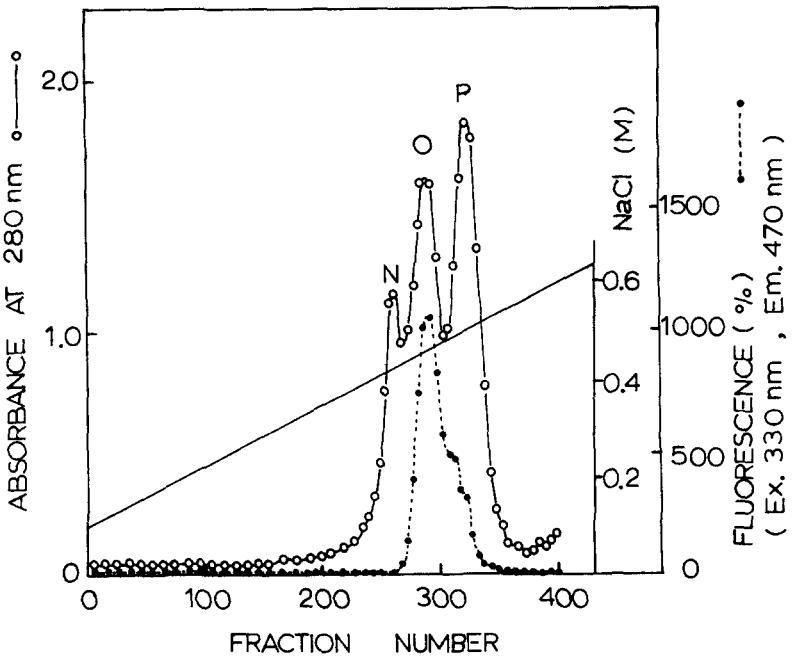

Fig. 4. The third DEAE-Sephadex column chromatography of a portion of the RBP pool (peak M) obtained after the second DEAE-Sephadex chromatography (see Fig. 3). The sample was concentrated and dialyzed exhaustively against 0.02 M potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The dialyzed sample (70 ml, containing 1.5 g of protein) was applied to a column (2.5 × 99 cm) of DEAE-Sephadex equilibrated with the same buffer. Protein was eluted with a linear gradient of NaCl from 0.1 to 0.6 M. Fractions of 20 ml were collected at a flow rate of 30 ml/hr. The RBP-containing fractions were eluted with peak O, and middle portions (fractions 280–291) were combined for further purification. Isolation of pure RBP–PA complex was accomplished by chromatography of this pool on Sephadex G-100.

into three peaks (peaks N–P) by a linear gradient elution using a long DEAE-Sephadex column as shown in **Fig. 4.** Protein-bound retinol was obtained as a single peak (peak O). The eluates from the middle portion of this peak were combined, concentrated, and dialyzed against 0.02 M potassium phosphate buffer, pH 7.0, 0.2 M NaCl. The sample was applied to a column (2.5 × 60 cm) of Sephadex G-100 equilibrated with the same buffer. Small amounts of nonfluorescent proteins with lower and higher molecular size than RBP–PA complex were removed. This final preparation was used for physicochemical studies, and it was also used for further isolation of RBP and PA as described below.

Isolation of RBP and PA. As reported elsewhere (5), the human RBP–PA complex can be dissociated into RBP and PA under conditions of low ionic strength, such as with 2 mM Tris–HCl buffer, pH 8.0. However, the application of this procedure to pure chicken RBP–PA complex resulted in only a partial dissociation. The most effective dissociation of the complex was achieved by CM-Sephadex column chromatography as indicated in **Fig. 5.** A portion of the highly purified RBP–PA complex (about 20 mg of protein) was dissociated into two distinct peaks: peak Q, which was without fluorescence, and peak R, which gave the strong fluorescence of RBP. Repeated separations were made with the same CM-Sephadex column chromatography procedure. The respective fractions (RBP and PA) were pooled and then their physicochemical properties were examined.

The yield in each purification step of RBP is shown in **Table 1.**

Purity of RBP–PA complex, RBP, and PA

The purified RBP–PA complex was also dissociated by disc gel electrophoresis into two protein bands; the faster-migrating component had no detectable fluorescence whereas the other was found to be fluorescent, as shown in **Fig. 6.** These two proteins were identified as PA and RBP, respectively, from the following results. When a portion of protein peak Q (see Fig. 5) was subjected to

TABLE 1. Purification of chicken RBP with column chromatography

Fraction	RBP	Ratio	Yield
	μg/mg protein[a]		%
Whole serum	0.43	1.0	100.0
1st DEAE-Sephadex	8.70	20.1	86.7
2nd DEAE-Sephadex	20.60	48.0	79.2
3rd DEAE-Sephadex	303.00	768.0	49.8
CM-Sephadex	1000.00	2330.0	34.8

[a] Measured by a single radial immunodiffusion.

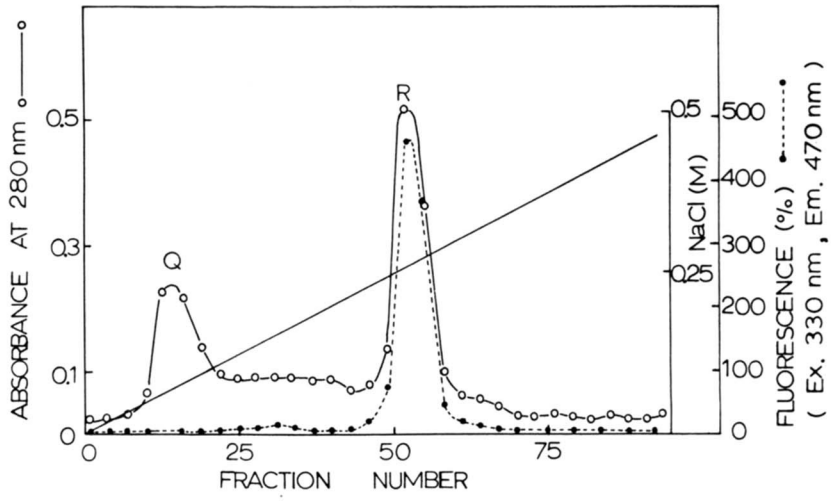

Fig. 5. Dissociation of a highly purified RBP–PA complex by CM-Sephadex column chromatography. The RBP–PA complex obtained after the third DEAE-Sephadex chromatography (see Fig. 4) and the subsequent gel filtration of Sephadex G-100 was dialyzed against 0.05 M acetate buffer, pH 5.4. The sample was applied to a column (2 × 20 cm) of CM-Sephadex equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl from 0 to 0.5 M. Fractions of 2 ml were collected at a flow rate of 15 ml/hr. RBP was detected by absorbance at 330 nm and by fluorescence with excitation and emission maxima at 330 and 470 nm, respectively. Peak Q (fractions 9–25) represented the portion containing PA, and peak R (fractions 48–60) had a strong fluorescence of RBP.

disc gel electrophoresis, a single protein band was observed, migrating faster than albumin and showing no fluorescence. Hence, the protein band with faster mobility in Fig. 6 should be PA. On the other hand, a portion of peak R (see Fig. 5) migrated as a single fluorescent band. Therefore, the slower-migrating protein as presented in Fig. 6 should be the retinol-containing protein (RBP).

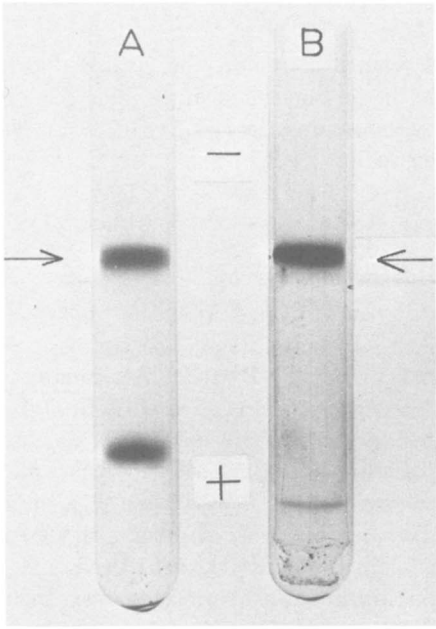

Fig. 6. Polyacrylamide gel electrophoresis of chicken RBP–PA complex (*A*) and chicken RBP (*B*) in Tris–glycine buffer at pH 8.9. The arrows indicate the fluorescence of retinol.

Molecular weights of RBP–PA complex, RBP, and PA

The molecular weight of the RBP–PA complex was estimated to be 76,000 by gel filtration on Sephadex G-200 (**Table 2**). In addition, the sedimentation velocity study showed that the purified complex migrated as a single homogeneous protein with a sedimentation constant of 5.2 S, whereas the sedimentation constant of purified RBP was calculated to be 2.0 S. The molecular weight of the RBP was also found to be 20,000 by gel filtration on Sephadex

TABLE 2. Characterization of chicken serum RBP, PA, and RBP–PA complex

	RBP	PA	RBP–PA Complex
Molecular weight			
By gel filtration (Sephadex)	20,000 (G-75)	56,000 (G-100)	76,000 (G-200)
By SDS electrophoresis	19,000	13,000 (subunit)	19,000 + 13,000
By sedimentation equilibrium	20,500	52,600	
By amino acid analysis	21,700	54,800 (subunit, 13,700)	76,500
Sedimentation velocity ($s_{20,w}$)	2.0 S	3.9 S	5.2 S
Partial specific volume[a]	0.713	0.727	0.725
$E^{1\%}_{1cm}$ 280 nm	19.2	22.8	20.2
Ratio of 330 nm to 280 nm	1.07	0	0.57
Carbohydrate			
Hexosamine	Nil	Nil	Nil
Neutral carbohydrate	Nil	Nil	Nil

[a] Calculated from amino acid composition.

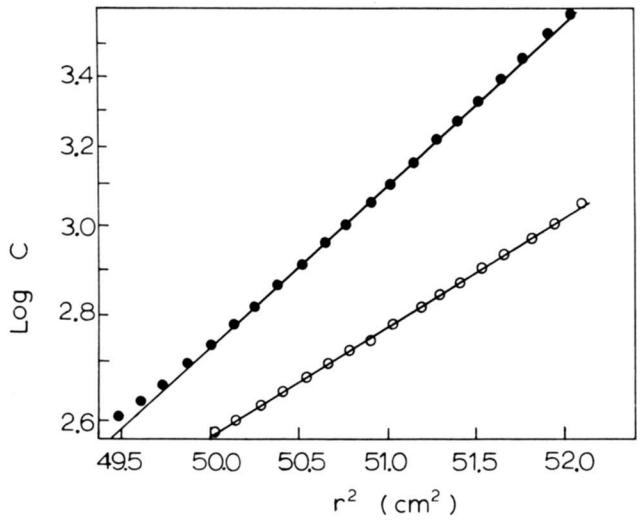

Fig. 7. Sedimentation equilibrium analyses of purified RBP (*open circles*) and PA (*closed circles*). *r* represents the distance from the axis of rotation. The concentration (*C*) is expressed in arbitrary units. RBP was centrifuged at 20,410 rpm and PA at 11,270 rpm.

G-75 and 19,000 by SDS–disc gel electrophoresis. In addition, the molecular weight of the RBP was calculated to be 20,500 from sedimentation equilibrium analysis (**Fig. 7**). Considering all the data, the RBP appears to be a single-chain polypeptide with a molecular weight of approximately 20,000.

The molecular weight of the PA was estimated to be 56,000 by gel filtration on Sephadex G-100 and 52,600 by sedimentation equilibrium analysis (Fig. 7). The sedimentation constant was calculated to be 3.9 S. However, the molecular weight of purified PA was found to be 13,000 by SDS–disc gel electrophoresis. From the results of gel filtration, sedimentation equilibrium analysis, and SDS–disc gel electrophoresis, the chicken PA should have a structure of four subunits, each with a molecular weight of about 13,000.

Disc gel electrophoresis of RBP and PA

As mentioned earlier, the RBP–PA complex separated into two protein bands on disc gel electrophoresis and the RBP showed a single protein band overlapping with a single fluorescent band (see Fig. 6). Moreover, the fresh RBP obtained by CM-Sephadex column chromatography migrated with exactly the same mobility as that of complexed RBP (Fig. 6). When this RBP preparation was kept at −10°C for 3 months, two protein bands were observed on disc gel electrophoresis; the slower-migrating band was still fluorescent (holo-RBP), whereas the slightly faster one gave no fluorescence (apo-RBP). The aged RBP was also denatured with 8 M urea and then subjected to urea-containing disc gel electrophoresis by the method of Möller and Chrambach (11). The migrating material had no fluorescence and appeared to have only one protein band. Hence, it is now conceivable that the chicken RBP molecule migrates as a single fluorescent band in the α region without any heterogeneity on electrophoresis, unlike human and rat RBP.

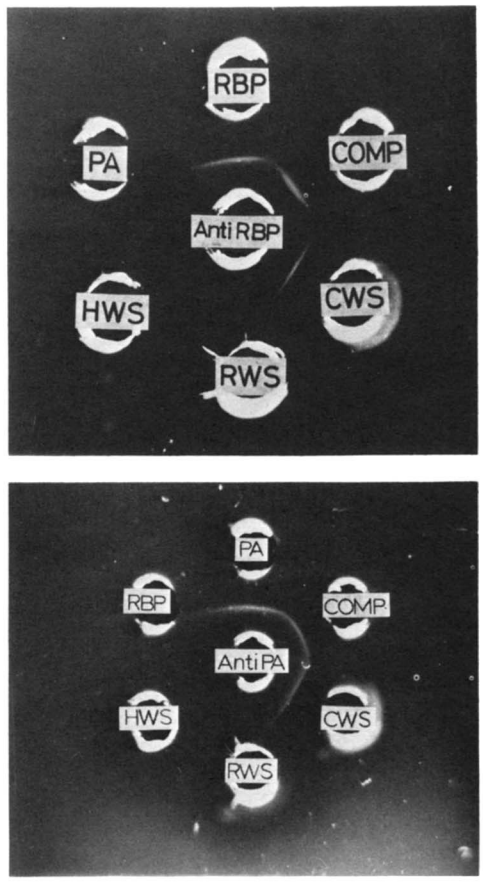

Fig. 8. Immunodiffusion of purified RBP, PA, RBP–PA complex (COMP), chicken whole serum (CWS), rat whole serum (RWS), and human whole serum (HWS). These were tested to rabbit antisera against chicken RBP (*top*) and chicken PA (*bottom*). These antisera gave a single precipitin line.

The chicken PA migrated as a single band as shown by electrophoresis on disc gels with or without SDS.

Immunological studies

The antiserum obtained from the rabbit injected with pure chicken RBP gave a single precipitin line when tested against purified RBP, RBP–PA complex (COMP), and chicken whole serum (CWS) (**Fig. 8,** *top*). Moreover, a complete fusion of the precipitin lines was observed. No immunoprecipitin lines were obtained when the antiserum was tested against purified chicken PA, human whole serum (HWS), and rat whole serum (RWS). Using the antiserum against the pure chicken PA (Fig. 8, *bottom*), a single precipitin line was obtained against purified chicken PA, RBP–PA complex, and chicken whole serum, indicating immunological identity. No precipitin lines were observed against purified RBP, human whole serum, and

TABLE 3. Amino acid compositions of chicken RBP and PA

Amino Acid	RBP Residues per Molecule[a] Average	RBP Residues per Molecule[a] Nearest Integer	PA Residues per Subunit[a] Average	PA Residues per Subunit[a] Nearest Integer	PA Residues per Molecule
Lysine	14.50	15	7.52	8	32
Histidine	3.49	3	4.73	5	20
Arginine	10.16	10	3.63	4	16
Aspartic acid	31.10	31	9.64	10	40
Threonine	8.80	9	8.62	9	36
Serine	12.40	12	9.10	9	36
Glutamic acid	18.70	19	11.61	12	48
Proline	4.40	4	2.35	2	8
Glycine	14.20	14	10.28	10	40
Alanine	13.20	13	11.16	11	44
Half-cystine[b]	3.00	3	1.00	1	4
Valine	13.90	14	11.82	12	48
Methionine	2.77	3	1.32	1	4
Isoleucine	3.32	3	2.30	2	8
Leucine	9.88	10	7.24	7	28
Tyrosine	6.55	7	3.08	3	12
Phenylalanine	10.45	10	6.58	7	28
Tryptophan[c]	5.82	6	3.08	3	12
Total		186		116	464

[a] Calculations for RBP and PA were based on assumed presence of three and one half-cystine residues, respectively.
[b] Measured as cysteic acid after performic acid oxidation.
[c] Determined spectrophotometrically according to the method of Edelhoch (16).

rat whole serum. On immunoelectrophoresis, each antiserum also yielded a single precipitin arc when tested against pure RBP or PA, RBP–PA complex, and chicken whole serum. These results indicate that each antiserum is monospecific for either RBP or PA, regardless of the molecular (free or complexed) forms of RBP and PA. Hence, purified RBP and PA preparations are both highly pure by immunological as well as physical criteria.

Spectral studies of RBP–PA complex, RBP, and PA

As reported elsewhere (1–5), the absorption spectrum of the chicken RBP also gave two peaks, with maxima at 280 nm and 330 nm. The absorbance ratios (330 nm/280 nm) were found to be 1.07 for the RBP, 0.57 for the RBP–PA complex, and nil for the PA (see Table 2). Fluorescence spectra (corrected) were recorded under several conditions for the three purified proteins. The RBP–PA complex and RBP gave the same fluorescence spectrum. When these proteins were excited at 330 nm, a peak of emission was recorded with maximum at 470 nm. The excitation spectrum measured for these proteins with emission at 470 nm gave two maxima, at 280 nm and 330 nm. The emission spectrum had two major peaks with maxima at 330 nm and 470 nm when these proteins were excited at 280 nm. The first peak (330 nm) presumably represents the emission spectrum of the protein itself (particularly its tryptophan and tyrosine residues; see Table 3), whereas the peak at 470 nm represents the emission spectrum of retinol bound to the RBP. The emission and excitation spectra indicate that there is efficient transfer of energy within the RBP molecule, contributed by excited tryptophan residues to the protein-bound retinol.

The extinction coefficients ($E^{1\%}_{1cm}$ 280 nm) were estimated to be 19.2 for RBP, 20.2 for RBP–PA complex, and 22.8 for PA.

The above-mentioned physicochemical properties of these purified proteins are summarized in Table 2.

Amino acid analyses

Amino acid analyses of the purified chicken RBP and PA are given as residues per molecule after 24, 48, and 72 hr of acid hydrolysis (**Table 3**). The contents of lysine, aspartic acid, glycine, and valine were found to be higher in chicken RBP than in human RBP (1, 2). The chicken RBP and PA also had a high content of tryptophan. For the chicken PA, residues of half-cystine, methionine, and tryptophan were calculated to be the same as those of human (2), monkey (26), and rat (27) PA.

Sugar content

Negligible amounts of carbohydrate in purified chicken RBP and PA were observed by the orcinol method (24). Likewise, no hexosamines were detected in these proteins during amino acid analyses.

Peptide maps of RBP–PA complex, RBP, and PA

The denatured (see Experimental Procedure) RBP–PA complex, RBP, and PA preparations were treated with trypsin for 22 hr. The denatured proteins are about 80% digested with this treatment (17). After two-dimensional peptide mapping using ascending development, the chicken RBP–PA complex yielded 14 fluorescent spots by phenanthrenequinone staining (**Fig. 9**). The chicken RBP and PA showed 10 and 4 fluorescent spots, respectively. All fluorescent spots were also stained by ninhydrin. As shown in Fig. 9, these spots of RBP and PA did not overlap each other. Moreover, these 14 spots of RBP plus PA were identical with 14 spots obtained with the RBP–PA complex. By disc gel electrophoresis studies and molecular weight estimations, the RBP was found to exist as a single polypeptide structure. The amino acid composition of RBP indicated that there are 10 arginine residues per molecule (Table 3). The chicken PA, however, was considered to have a subunit structure of four polypeptides. These four polypeptides had equivalent molecular weights of 13,000 by SDS–disc gel electrophoresis. Therefore, it was estimated that one PA molecule, which contained 16 arginine residues by amino acid analysis, should have 4 arginine residues in each subunit. The finding of four fluorescent spots in peptide mapping of the PA (Fig. 9) strongly supports the conclusion that the chicken PA contains four identical polypeptide chains.

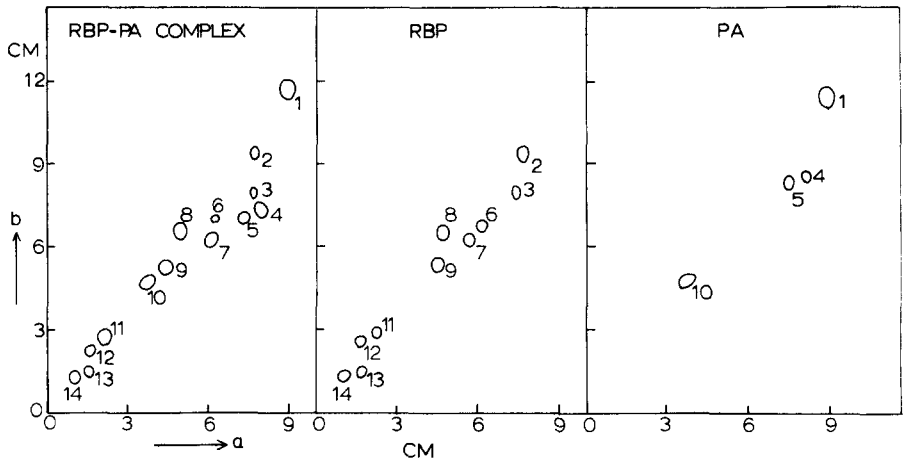

Fig. 9. Peptide maps after tryptic digestion of purified chicken RBP, PA, and RBP–PA complex. First direction (*a*), in *n*-butanol–acetic acid–water 4:1:2 (v/v); second direction (*b*), in *n*-butanol–acetic acid–pyridine–water 5:1:4:4 (v/v). The schematic drawings represent phenanthrenequinone-positive spots of RBP–PA complex, RBP, and PA. For details see Experimental Procedure.

DISCUSSION

The present study clearly demonstrates that vitamin A in chicken plasma is bound to a specific protein, retinol-binding protein, similar to what has been observed in man (1, 2), monkey (26), and rat (3, 27). Chicken RBP migrates as an α-globulin on electrophoresis and has a sedimentation constant of 2.0 S and a molecular weight of approximately 20,000 (see Table 2). These findings have been generally confirmed by the recent report of Mokady and Tal (7).

The principal aim of this study, however, was to isolate a highly pure RBP–PA complex from chicken plasma, because vitamin A-containing protein in fresh plasma had an obviously larger molecular size (70,000–80,000) than that of the RBP finally purified. The isolation of the complex proved to be technically difficult, much more so than in the case of human RBP–PA complex, mainly because of the incomplete separation from albumin even under several different chromatographic conditions. However, a third chromatographic separation on a long DEAE-Sephadex column was found to be effective (Fig. 4). The highly purified chicken RBP–PA complex has a sedimentation constant of 5.2 S and a molecular weight of approximately 76,000, which is in agreement with a molecular size of vitamin A-containing protein in native plasma. The results, in turn, provide additional direct evidence that chicken RBP circulates as a 1:1 molar protein–protein complex with PA, as the molecular weight of purified PA is estimated to be about 54,000 (Table 2).

Dissociation of the RBP–PA complex into each component was effected by CM-Sephadex column chromatography (pH 5.4) as shown in Fig. 5. The preparations of isolated chicken RBP and PA used for physicochemical studies were highly pure by physical and immunological criteria.

The physicochemical properties of chicken RBP are similar in many ways to those of human (1, 2) and rat (3, 27). These proteins have virtually identical ultraviolet absorption spectra and fluorescence emission and excitation spectra. Studies with human RBP have shown that the absorption peak at 330 nm represents the absorption of protein-bound retinol, that the molecular extinction of retinol bound to RBP is identical with that of retinol in benzene solution, and that the RBP molecule appears to have a single binding site for one molecule of retinol (1). It is likely that these statements also apply to the chicken RBP, which was shown to be highly saturated with retinol (the ratio of 330 nm to 280 nm was 1.07).

In contrast to human and rat RBP, the purified chicken RBP showed no microheterogeneity on disc gel electrophoresis. As shown in Fig. 6, the fresh preparation of chicken RBP migrated as a single protein band with strong fluorescence in the α region, which probably corresponds with H2 of human and rat RBP (1, 3) or B2 of human RBP (2). When chicken RBP is aged, however, an additional band without fluorescence (apo-RBP) was observed to migrate with a slightly faster mobility. In any event, chicken RBP appears to be much more stable than RBP of man and rat, in terms of microheterogeneity on disc gel electrophoresis. The results suggest that certain important structural or compositional differences may exist between chicken RBP and RBP from man and rat.

Moreover, chicken RBP is immunologically completely distinct and different from human and rat RBP (Fig. 8). Hence, it is also evident that certain important structural differences must exist among these three proteins, particularly with regard to those aspects of structure involved in the antigenic determination.

The amino acid composition of chicken RBP, however, is similar to that of human (1, 2) and rat (3, 27) RBP. In general, RBP has a high content of aromatic amino acid residues, and this has been confirmed by Mokady and Tal (7). The major quantitative differences among these three proteins, however, were the higher levels of lysine, aspartic acid, and glycine found in the chicken RBP (Table 3); other smaller differences were also observed with regard to several amino acid residues.

The chicken PA is the major thyroxine-binding protein in blood (28). The molecular weight of chicken PA, which has not been isolated previously, was estimated to be approximately 54,000 (Table 2). In other vertebrate species, the molecular weight of PA has been reported to be 54,000 in man (4), 51,000 in rat (3, 27), and 58,000 in monkey (26). Hence, the molecular weight of chicken PA is found to be similar to that of PA from some mammalian vertebrates. Moreover, the chicken PA consists of four subunits, as has been found for PA of other vertebrates (29–32). Amino acid analysis of the chicken PA, however, showed amounts of threonine and proline to be higher than those found in human (2, 4) and monkey (26) PA. Chicken PA is also immunologically distinct from both human and rat PA. Studies on thyroxine binding with PA are underway in this laboratory.

The chicken RBP–PA model has two obvious major advantages: large amounts of blood can be easily obtained for purification, and vitamin A metabolism in the chick embryo and organ culture system warrants further exploration with respect to the metabolism of RBP and PA, since better preservation can be achieved in chick embryo than in other species such as man and rat.

We are grateful to Dr. Kunio Yamane for his helpful assistance with the amino acid analyses and peptide mapping. We also would like to express our thanks to Dr. Kazuo Sibata for the fluorescence assays and to Drs. Katsuji Takai and Arata Ichiyama for their helpful suggestions and constructive criticism. This work was partially supported by US–Japan Cooperative Research Program grant 5R037 from the Japan Society for Promotion of Science.

Manuscript received 13 September 1974; accepted 17 January 1975.

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