Transport of retinol in the duck plasma

B. SRIDHARA RAO, N. APPAJI RAO and H. R. CAMA

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012

Abstract. Retinol-binding protein and prealbumin were isolated from duck plasma by chromatography on DEAE-cellulose-and DEAE-Sephadex A-50, gel filtration on Sephadex G-100 and preparative Polyacrylamide gel electrophoresis. The molecular weights of the retinol-binding protein-prealbumin complex, prealbumin and retinol-binding protein were found to be 75,000, 55,0000 and 20,000, respectively. On sodium dodecyl sulphate Polyacrylamide gel electrophoresis, prealbumin dissociated into identical subunits exhibiting a molecular weight of 13,500. Retinol-binding protein exhibited microheterogeneity on electrophoresis, whereas prealbumin moved as a single band unlike the multiple bands observed in chicken and rat. The ultraviolet and fluorescence spectra of the two proteins were similar to those isolated from other species. No carbohydrate moiety was detected in either retinol-binding protein or prealbumin. Duck retinol-binding protein and prealbumin showed cross-reactivity with their counterparts in chicken but differed immunologically from those of goat and man. Retinol-binding protein and prealbumin could be dissociated at low ionic strength, in 2M urea, by CM-sephadex chromatography or on preparative electrophoresis. Although the transport of retinol in duck plasma is mediated by carrier proteins as in other species, it is distinguished by the absence of microheterogeneity in prealbumin and of an apo-retinol-binding protein form that could be transported in the plasma.

Keywords. Retinol; prealbumin; plasma transport; duck.

Introduction

It is well established that retinol is transported in human plasma bound to a specific retinol-binding protein (Kanai *et al.,* 1968). The retinol-binding protein forms a tight complex with plasma prealbumin and normally circulates as an equimolar complex with it (Peterson and Berggard, 1971; Raz and Goodman, 1969; Peterson, 1971). A similar retinol-transport system has been reported in several other species (Muto *et al.,* 1973; Peterson *et al.,* 1974; Abe *et al.,* 1975; Bhat *et al.,* 1977) and evidence has been presented for the identity of the plasma and egg-yolk retinol-binding protein-prealbumin complexes in the case of chicken (Heller, 1976). In pursuance of earlier studies in our laboratory on the mode of transport of retinol for embryonic development in duck (Sreekrishna and Cama, 1978), the plasma transport system in duck was characterized by the isolation of retinol-binding protein and prealbumin.

Materials and methods

Materials

Blood was collected after decapitation of the ducks and was allowed to clot. The clear supernatant serum was collected and processed immediately. DEAE-cellulose (0.9 milliequivalents/g), Sephadex G-100, bovine serum albumin, ovalbumin, cytochrome C, α-chymotrypsinogen, hexokinase, acrylamide, N,N,N',N'-tetramethylene diamine, ammonium persulphate, bromophenol blue, Coomassie Brilliant Blue, Tris (hydroxymethylarninomethane) and glycine were all purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Agar and Freund's complete adjuvant were obtained from Difco Lab., Detroit, Michigan, USA. All other chemicals were of reagent grade.

Methods

Protein-bound retinol was monitored by measuring the relative fluorescence intensity (F) at 470 nm upon excitation at 330 nm in a Perkin Elmer spectrofluorometer (Model 203). Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard. Analytical Polyacrylamide gel (7.5% w/v) electrophoresis was performed by the method of Davis (1964). Glyco-protein staining was performed according to the method of Segrest and Jackson (1972). Preparative electrophoresis was carried out using cylindrical gels (1.2×9 cm) with 0.5-1.0 ml of spacer gel. After electrophoresis, the gel was examined under an ultraviolet lamp for fluorescence of protein-bound retinol. The fluorescent zone was sliced and retinol-binding protein eluted from the gel slice by electro-elution or by homogenization and extraction with 20mM Tris-HCl buffer, pH 7.6 containing 0.2M NaCl. The gel particles were removed either by centrifugation at 12,000 *g* for 10 min or by filtration through a Whatman No. 1 filter paper. For prealbumin, the region of the gel between the fluorescent zone and the marker dye was cut into thin slices and the protein eluted from each slice as described above. The single protein peak obtained with a mobility intermediate between the marker dye and albumin was designated as prealbumin. Occassionally, the prealbumin band was identified by comparison with a duplicate gel stained with Coomassie Brilliant Blue G.

Determination of molecular weight. Molecular weights were determined by gel filtration using a Sephadex G-100 column calibrated with cytochrome C, α-chymotrypsinogen, ovalbumin, bovine serum albumin and hexokinase according to the method of Whitaker (1963). Sodium dodecyl sulphate Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using bovine serum albumin, ovalbumin and cytochrome C as standards.

Preparation of antibodies. Rabbits (Institute strain) were immunized by intradermal injection, once a week, with 100 μg of retinol-binding protein or prealbumin in phosphate buffered saline emulsified in Freund's complete adjuvant. After three immunizations, a booster injection of 300 μg of protein in phosphate buffered saline was given and after six days the animals were bled through the marginal ear vein, the serum prepared and stored frozen. Immuno-double diffusion was carried out according to the method of Ouchterlony (1967).

Binding experiments. The binding of thyroxine and retinol-binding protein to prealbumin was monitored by the quenching of the protein fluorescence of pre-albumin. The control employed in studying thyroxine binding was N-acetyl-L-tryptophanamide having the same absorbance as that of prealbumin. When the binding of retinol-binding protein was being monitored, the increase in the fluorescence upon addition of retinol-binding protein to buffer alone was deducted from the experiment.

The dissociation-association experiments were carried out by gel filtration on a Sephadex G-100 column (0.9×38 cm).

Preparation of apo-retinol-binding protein. Apo-retinol-binding protein was prepared by shaking retinol-binding protein solution with an equal volume of diethyl ether at a rate of 75 ±5 strokes per min in the dark at 25 ±2°C for 6 h, under nitrogen in a stoppered test tube.

Results

Purification

Duck serum was applied onto a DEAE-cellulose column and the bound protein were eluted by a linear gradient of sodium chloride concentration from 0.1 to 0.35M. Protein-bound retinol eluted on the descending side of the main protein peak (figure 1). The fluorescent fractions were pooled, dialyzed against 0.1 M
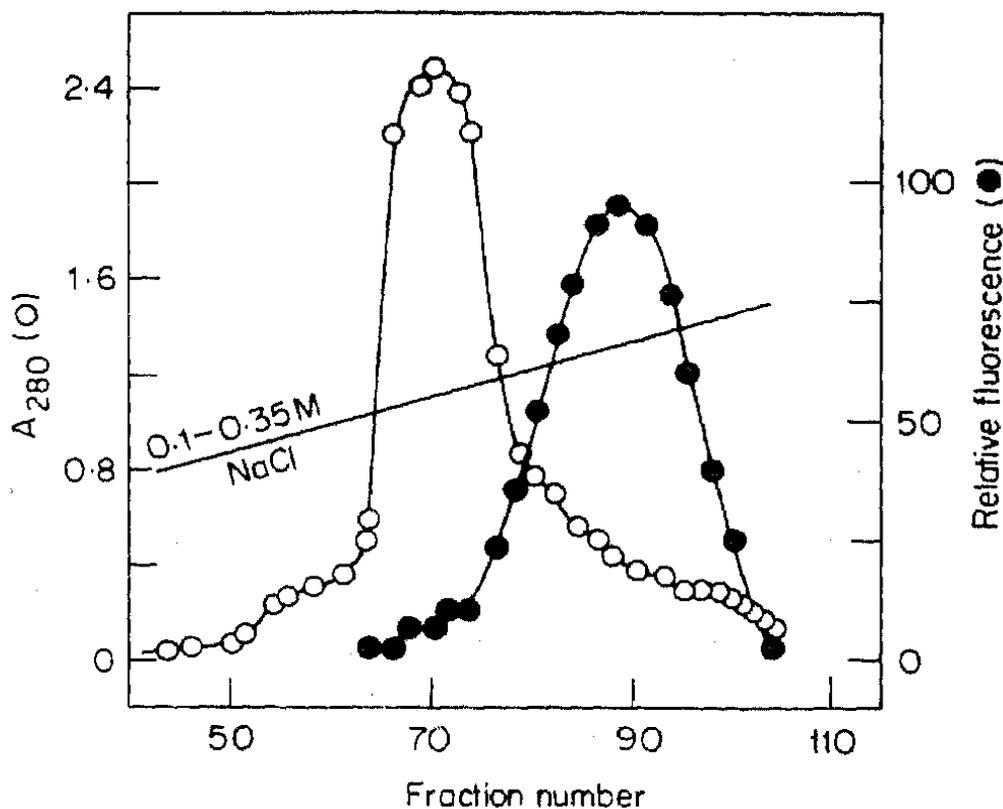

Figure 1. DEAE-cellulose chromatography of duck serum. Serum (100ml) dialyzed against 20 mM Tris-HCl buffer pH 7.6, containing 0,1 M NaCl was loaded onto a column (2×50 cm) of DEAE-cellulose equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of sodium chloride from 0.1 to 0.35 M, Fractions of 10 ml were collected at a flow rate of 30'ml/h. Fluorescent fractions were pooled.

sodium acetate buffer, pH 5.4, and subjected to a second ion exchange chromato-graphy on DEAE-Sephadex. Elution was carried out by a linear gradient of 0.1 to 0.5M sodium acetate. Sephadex G-100 gel filtration of the fluorescent fraction from the above step resulted in the elution of two protein peaks (figure 2). The
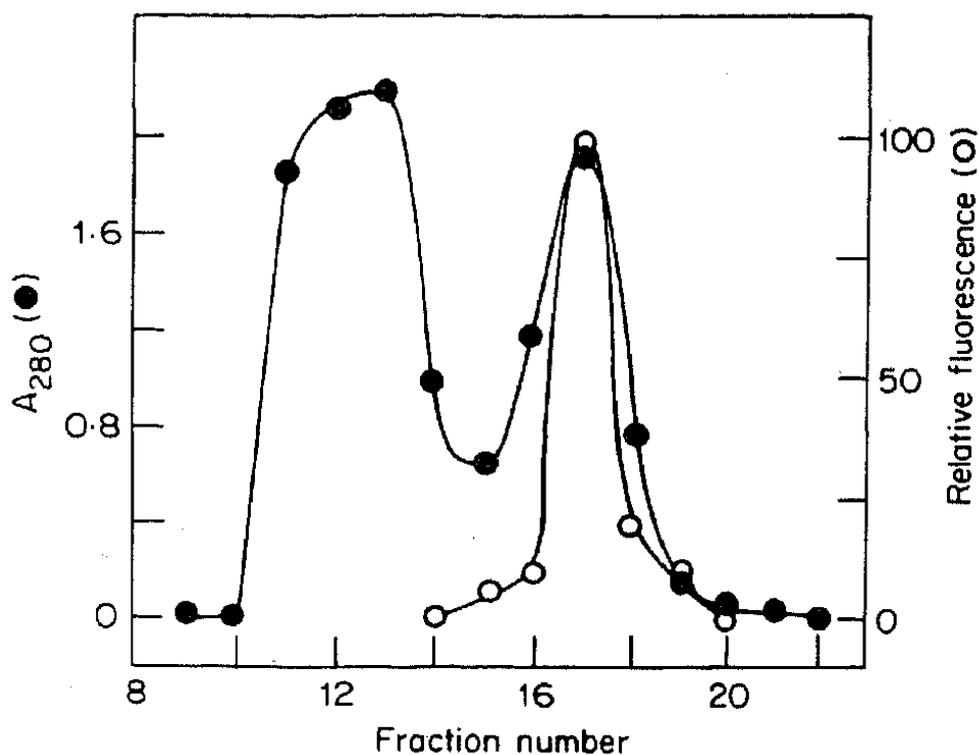

Figure 2. Sephadex G-100 gel filtration. The fluorescent fraction from the ion exchange chromatography was concentrated, dialyzed against 20 mM Tris-HCl buffer pH 7.6, containing 0.2M NaCl and applied on a column (2×44 cm) of Sephadex G-100 equilibrated with the same buffer. Fractions of 3 ml were collected at a flow rate of 12 ml/h.

fluorescence was associated with the second peak. These fractions were pooled, concentrated and subjected to preparative polacrylamide gel electrophoresis. Localization of proteins and elution was carried out as described under Methods. Retinol-binding protein, after this step, was occasionally found to be contaminated with traces of albumin and hence was further purified by gel filtration on Sephadex G-100. The prealbumin obtained was pure and did not require further purifica-tion. Retinol-binding protein and prealbumin could be reassociated to form a complex (see below).

Electrophoretic pattern

On analytical polacrylamide gel electrophoresis, retinol-binding protein exhibited two closely spaced bands exhibiting greenish blue fluorescence characteristic of retinol. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that the two species had the same molecular weight (M_r 20,000). In analogy with

the existing nomenclature, the fast and slow moving bands were designated as holo$_f$- retinol-binding protein and holo$_s$-retinol-binding protein, respectively. Evidently, the amount of the fast moving spcies is much higher. Prealbumin exhibited a single band, with a much faster mobility, trailing just behind the marker dye (figure 3).
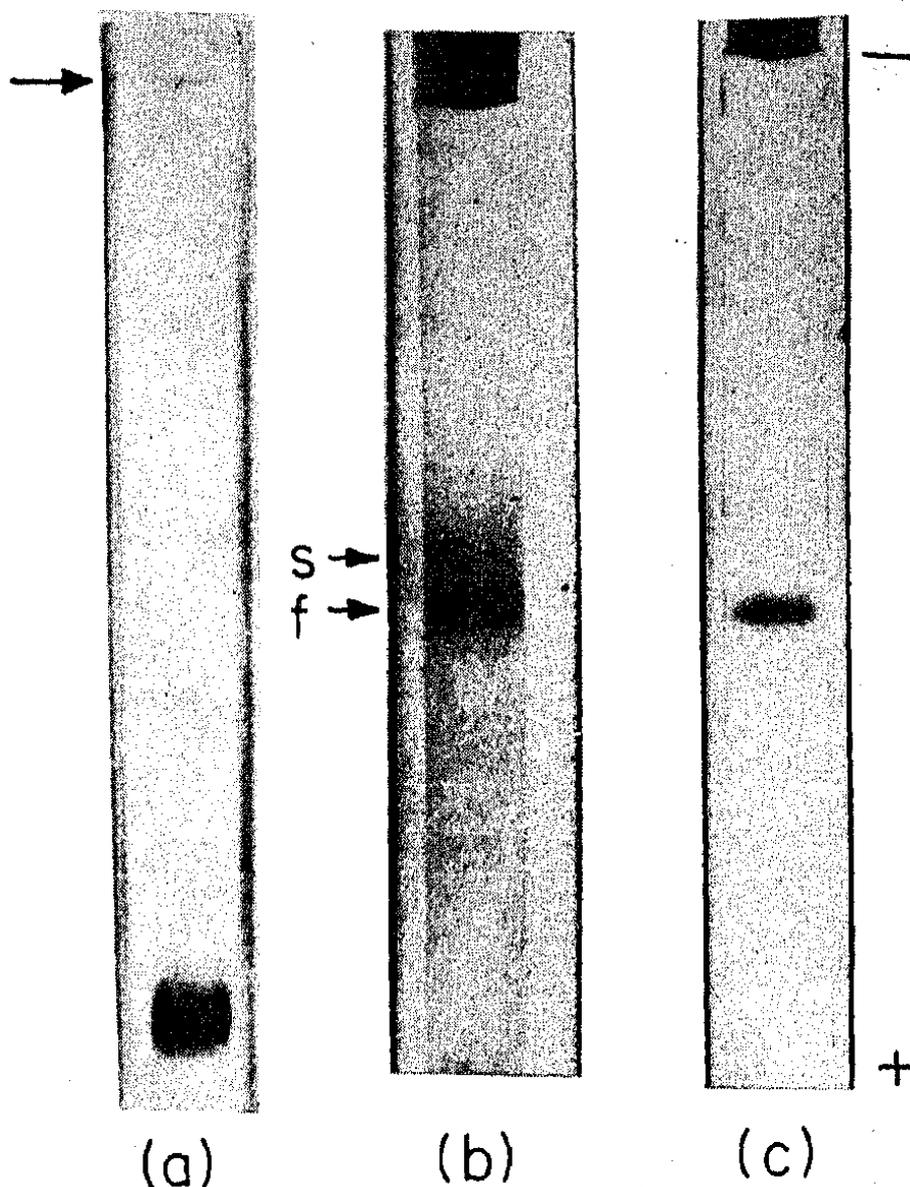

Figure 3. Electrophoretic pattern on 7.5% Polyacrylamide gels in Tris-glycine buffer at pH 8.3 (a) Prealbumin (b) Retinol-binding protein. s and f indicate holo$_s$- and holo$_f$-retinol-binding proteins, respectively, (c) Holo$_f$-retinol-binding protein separated by preparative Polyacrylamide gel electrophoresis.

Molecular weights

The molecular weights of complexed retinol-binding protein, prealbumin and retinol-binding protein, as estimated by gel filtration, were 75,000, 55,000 and 20,000, respectively. On sodium dodecyl sulphate Polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, retinol-binding protein exhibited a molecular weight of 20,000 while prealbumin dissociated into subunits of molecular weight, 13,500.

Spectroscopic properties

Retinol-binding protein exhibits two absorption maxima in the ultraviolet region, one at 280 nm and the other at 330 nm. The retinol-binding protein-prealbumin complex shows only a slight absorbance at 330 nm. The ratio of the absorbance at 280 nm to 330 nm was found to be 1.2 in the case of retinol-binding protein and 3.1 in the case of the retinol-binding protein-prealbumin complex. The fluorescence spectra of free as well as complexed retinol-binding protein exhibited two excitation maxima, one at 280 nm and the other at 330 nm and two emission maxima, one at 330 nm and the other at 470 nm. HoIo_s- and holof-retinol-binding proteins were both fluorescent and their fluorescence spectra were similar and overlapping.

Glycoprotein staining was found to be negative for retinol-binding protein as well as prealbumin.

The addition of retinol-binding protein or L-thyroxine to prealbumin quenched the protein fluorescence indicating that both L-thyroxine and retinol-binding protein could bind prealbumin (figure 4). Retinol-binding protein from which retinol was extracted by diethylether was not able to bind prealbumin.
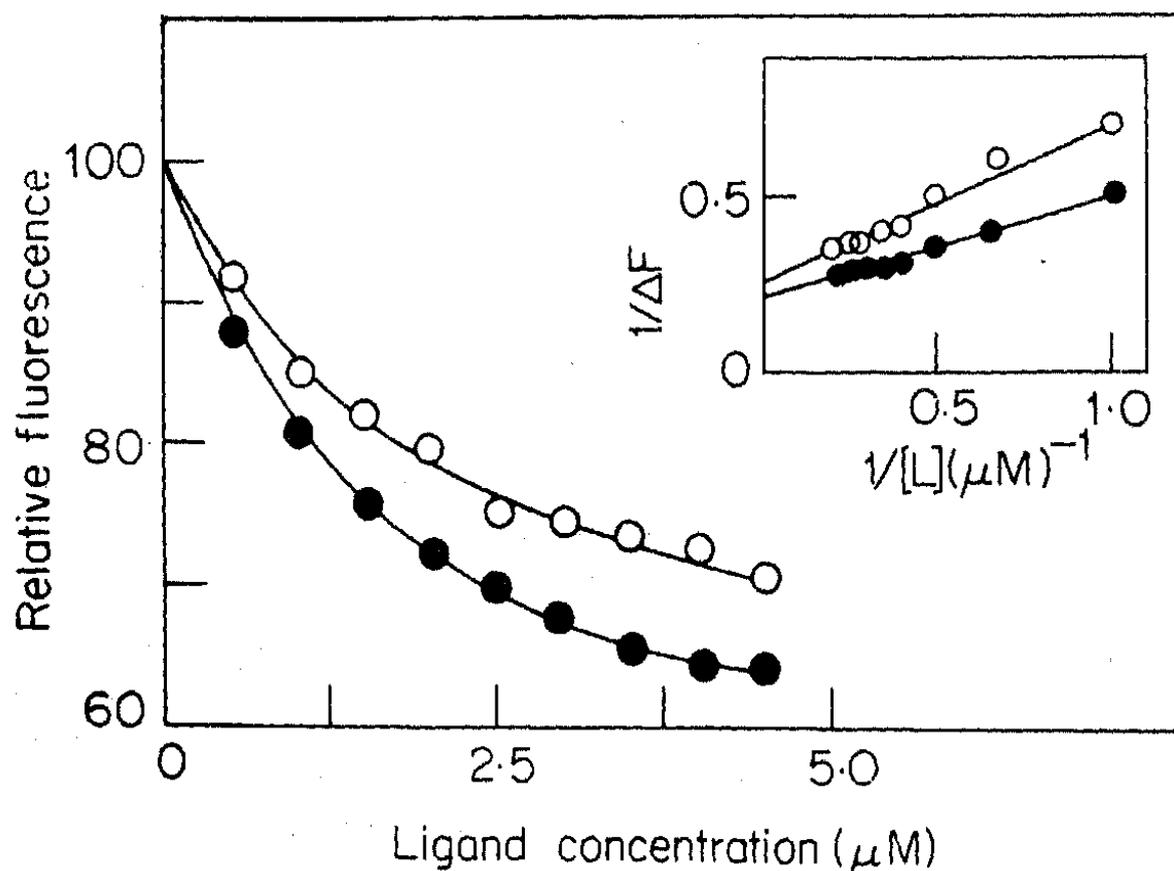

Figure 4. Quenching of the fluorescence of prealbumin by thyroxine and retinol-binding protein. Excitation and emission were set at 280 nm and 340 nm, respectively To 1 μM solution of prealbumin in 20 mM Tris-HCl buffer pH 7.6 containing 0.2M NaCl, aliquots of retinol-binding protein in the same buffer or L-thyroxine in 10mM NaOH were added. During titration with retinol-binding protein (O), the increase in fluorescence of the buffer alone on the addition of retinol-binding protein was deducted from the experiment. In the case of the titration with thyroxine (●), N-acetyl-L-tryptophanamide solution having the same absorbance as that of prealbumin was employed as blank. ΔF: Change in fluorescence. The ΔF_{max} obtained was 45 for thyroxine and 30 for retinol-binding protein

Dissociation and association

The effects of various factors on the stability of the retinol-binding protein-prealbumin complex were tested. When buffers of various ionic strengths were tested, it was found that a low ionic strength buffer i.e., 2mM Tris-HCl, pH 7.6, could completely dissociate the complex. The complex was found to be less susceptible to changes in pH in the range of 8 to 3. A negligible amount of retinol-binding protein was found to be dissociated at pH 3.0. Another effective method of dissociation was chromatography on CM-Sephadex at pH 5.4, when the prealbumin eluted earlier than retinol-binding protein (figure 5). The presence of 2M urea in the buffer could also bring about dissociation.
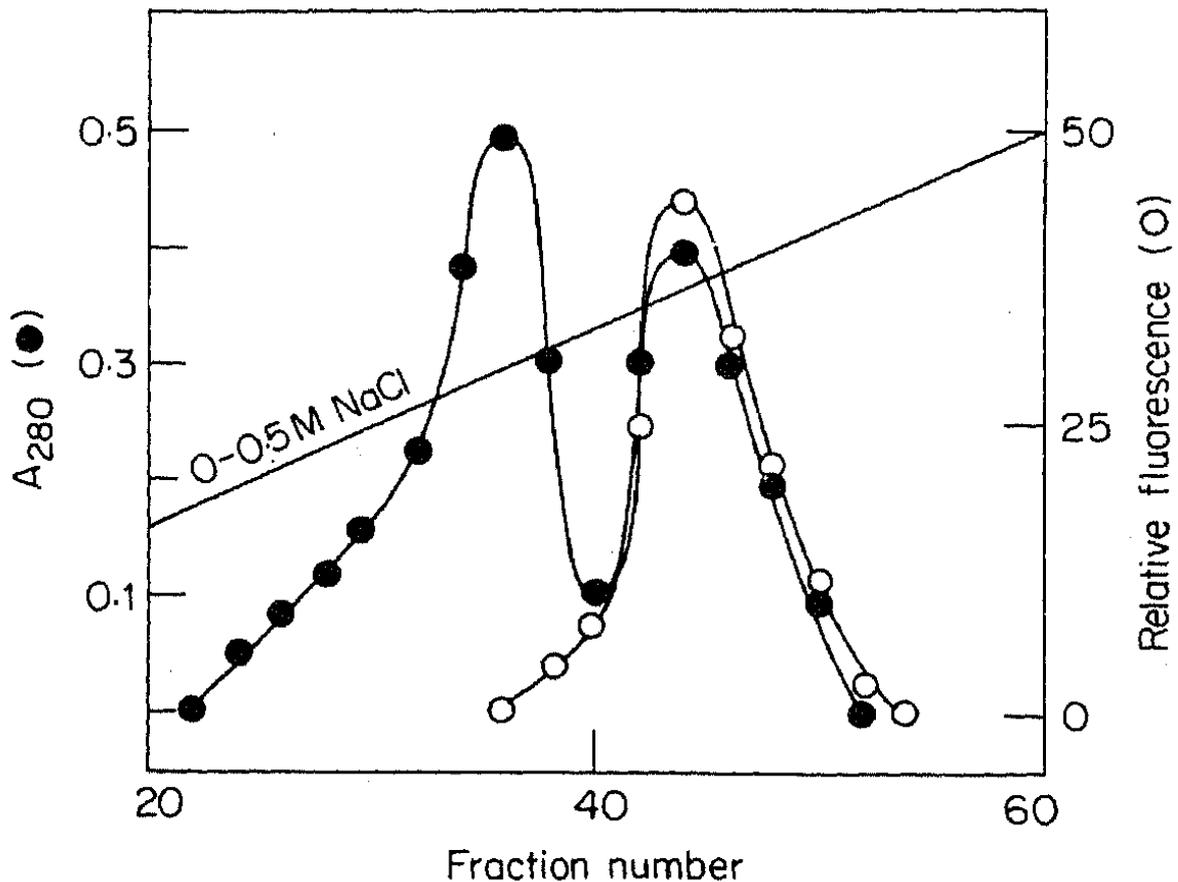

Figure 5. Dissociation of retinol-binding protein-prealbumin complex by CM-Sephadex chromatography. The complexed retinol-binding protein was dialyzed against 50 mM sodium acetate buffer, pH 5.4 and applied onto a column (1.4×49 cm) of CM-Sephadex equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl from 0 to 0.5M. Fractions of 5 ml were collected at a flow rate of 30 ml/h. Peak I was found to be prealbumin and peak II, retinol-binding protein by Polyacrylamide gel electrophoresis.

The dissociation brought about by any of the above methods (including electrophoresis) was completely reversible. When retinol-binding protein and prealbumin were mixed and applied on a Sephadex G-100 column, the elution profile showed a shift of retinol fluorescence to an elution volume corresponding to a molecular weight of 75,000. No association was observed when prealbumin and apo-retinol-binding protein were applied on the column.

Immunological cross-reactivity

Ouchterlony immuno-double diffusion studies showed that duck retinol-binding protein and prealbumin exhibited strong cross-reactivity with their counterparts in chicken but did not show any cross reactivity with those of heterologous species like man and goat.

Discussion

In aggreement with the results obtained from other animal species, the transport of retinol in duck plasma is mediated by a retinol-binding protein-prealbumin complex. The physico-chemical properties of duck retinol-binding protein and prealbumin compare with those from other sources to some extent.

Duck retinol-binding protein showed microheterogeneity upon electrophoresis exhibiting a slow and a fast moving species. Abe *et al.* (1975) found chicken retinol-binding protein to be devoid of microheterogeneity while others (Kopelman *et al.,* 1976) reported to the contrary. We found, in the case of duck, two closely spaced bands of holo-retinol-binding protein but not of the apo-retinol-binding protein.

The A_{280}: A_{330} ratio of the duck retinol-binding protein is lower than that obtained in goat (Sreekrishna and Cama, 1979) and canine (Muto *et al.,* 1973) sources and agree well with the values reported in chicken (Heller, 1976).

An important observation in this study was that duck prealbumin did not exhibit microheterogeneity while that from other sources like chicken (Bhat *et al.,* 1977) exhibited multiple bands on electrophoresis. Another major difference between the duck apo-retinol-binding protein and that isolated from other species (Rask *et al.,* 1971) was its inability to bind prealbumin.

These results, while highlighting the universality of the carrier-mediated retinol-transport mechanism, emphasize that. major differences exist in the physico-chemical and immunological properties of these proteins from several species.

Acknowledgements

References

Abe, T., Muto, Y. and Hosoya, N. (1975) *J. Lipid Res.,* **16**, 200.

Bhat, M. K., Sreekrishna, K. and Cama, H. R. (1977) *Indian J. Biochem. Biophys.,* **14,**125.

Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.,* **121,** 321.

Heller, J. (1976) *Develop. Biol,* **51**, 1.

Kanai, M., Raz, A. and Goodman, D. S. (1968) *J. Clin. Invest.,* **47**, 2025.

Kopelman, M., Mokady, S. and Cogan, U. (1976) *Biochim. Biophys. Acta,* **439,**442.

Laemmli, U. K. (1970) *Nature* (*London*), **227,** 680.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951)*J. Biol. Chem.,* **193,** 265.

Muto, Y., Smith, F. R. and Goodman, D. S. (1973) *J. Lipid. Res.,* **14,** 525.

Ouchterlony, O. (1967) in *Handbook of Experimental Immunology* ed. D. M. Weir (Oxford: Blackwell Publication) p. 655.

Peterson, P. A. (1971)*J. Biol. Chem.,* **246,** 34.

Peterson, P. A. and Berggard, I. (1971)*J. Biol. Chem..,* **246,** 25.

Peterson, P. A., Nilsson, S. F., Ostberg, L., Rask, L. and Vahlquist, A. (1974) *Vitam. Horm.,* **32**, 181.

Rask, L., Vahlquist, A. and Peterson, P. A. (1971)*J, Biol. Chem.,* **246,** 6638.

Raz, A. and Goodman, D. S. (1969)*J. Biol. Chem.,* **244,** 3230.

Segrest, J. P. and Jackson, R. L. (1972) *Methods Enzymol,* **Part B 28,** 57.

Sreekrishna, K. and Cama, H. R. (1978) *Indian J. Biochem. Biophys.,* **15,** 255.

Sreekrishna, K. and Cama, H. R. (1979) *Biochem. Biophys. Acta,* **581,** 136.

Whitaker, J. R. (1963) *Anal. Chem.,* **35**, 1950.