Interactions of All-trans-, 9-, 11-, and 13-cis-retinal, All-trans-retinyl Acetate, and Retinoic Acid with Human Retinol-binding Protein and Prealbumin*

Joseph Horwitz and Joram Heller
From the Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, California 90024

SUMMARY

The reconstitution of analogs of the native chromophore retinol (vitamin A) with apo-retinol-binding protein (apo-RBP) from human plasma was studied by chromatographic and spectroscopic techniques. All-trans- and 9-, 11-, and 13-cis-retinal combined with apo-RBP in a 1:1 molar ratio while only about 0.85 mole of all-trans-retinoic acid and 0.75 mole of retinyl acetate combined with 1.0 mole of apo-RBP. The various chromophores all bound to the same site on retinol-binding protein and were competitive inhibitors of each other's binding. Upon reconstitution the absorption peak of the chromophore shifted some 9 to 12 nm to the red with the retinal chromophores, 5 nm to the red with retinyl acetate, and 15 nm to the blue with retinoic acid (at pH 9).

All the chromophore-RBP complexes showed an induced (extrinsic) Cotton effect of the chromophore absorption band with a rotatory strength of the same order of magnitude as that of the free chromophore in aqueous detergent solutions. The absorption and CD spectra of retinoic acid-RBP were pH dependent, with both the absorption and CD chromophore peak decreasing in magnitude and shifting to the red upon a decrease in pH. The reconstitution of apo-RBP with the various chromophores led to a marked (more than 90%) quenching of the fluorescence of the inherent protein chromophores. Increasing the ionic strength of a retinal-RBP complex led to a shift of the absorption to the blue (7 nm with the 9-cis-retinal), a small decrease in the size of the absorption band, and marked decrease (up to 39% with 11-cis-retinal) of the rotatory strength. Increasing the ionic strength of retinoic acid-RBP solution led to a shift of 1 nm to the red and an increase of about 1% in the rotatory strength.

Reduction of retinal-RBP complexes with NaBH₄ showed that the retinal aldehyde function was free and was not covalently linked to the protein. This was also shown by illuminating frozen solutions of retinal-RBP complexes with linearly polarized light (photoselection) and measuring the resulting linear dichroism spectrum. The linear dichroism spectrum of illuminated retinal-RBP complexes was similar to that of the corresponding free retinal isomer in aqueous digitonin solution.

None of the retinal isomers-RBP and retinyl acetate-RBP complexes was bound to prealbumin (thyroxine-binding protein) at physiological ionic strength, whereas retinoic acid-RBP was bound to prealbumin under the same conditions as judged by gel filtration chromatography. On the other hand, the addition of prealbumin to retinal-RBP complexes at low ionic strength resulted in a shift to the blue of the absorption peak (8 nm with 9-cis-retinal-RBP). Increasing the ionic strength of a retinal-RBP and prealbumin solution resulted in a further shift to the blue (5 nm with 9-cis-retinal-RBP) and an increase in the area of the absorption band. Adding salt to a retinoic acid-RBP solution containing prealbumin resulted in a shift of 1 nm to the red and an increase in the absorption band.

It was concluded from these experiments that although the retinals, retinoic acid, and retinyl acetate bind to retinol-binding protein at the same site as the retinol isomers, the binding of these various chromophores resulted in a somewhat altered conformation of the reconstituted retinol-binding protein. This in turn made it impossible for the retinals- and retinyl acetate-RBP complexes to bind to prealbumin and led to the various subtle differences in spectroscopic behavior of the various chromophore-RBP complexes upon changes in ionic strength and interaction with prealbumin.

In the preceding paper of this series (1) we described the reconstitution of apo-retinol-binding protein from human plasma with four geometric isomers of retinol (vitamin A). The all-trans- and the 9-, 11-, and 13-cis-retinols give about the same degree of reconstitution with apo-RBP, namely, 0.9 to 0.95 molecule per molecule of protein. All occupy the same binding site, give an enhanced quantum yield of fluorescence as compared to the free retinol, show a 3- to 5-nm shift to the red of the resulting linear dichroism spectrum. The linear dichroism spectrum of illuminated retinal-RBP complexes was similar to that of the corresponding free retinal isomer in aqueous digitonin solution.

None of the retinal isomers-RBP and retinyl acetate-RBP complexes was bound to prealbumin (thyroxine-binding protein) at physiological ionic strength, whereas retinoic acid-RBP was bound to prealbumin under the same conditions as judged by gel filtration chromatography. On the other hand, the addition of prealbumin to retinal-RBP complexes at low ionic strength resulted in a shift to the blue of the absorption peak (8 nm with 9-cis-retinal-RBP). Increasing the ionic strength of a retinal-RBP and prealbumin solution resulted in a further shift to the blue (5 nm with 9-cis-retinal-RBP) and an increase in the area of the absorption band. Adding salt to a retinoic acid-RBP solution containing prealbumin resulted in a shift of 1 nm to the red and an increase in the absorption band.

It was concluded from these experiments that although the retinals, retinoic acid, and retinyl acetate bind to retinol-binding protein at the same site as the retinol isomers, the binding of these various chromophores resulted in a somewhat altered conformation of the reconstituted retinol-binding protein. This in turn made it impossible for the retinals- and retinyl acetate-RBP complexes to bind to prealbumin and led to the various subtle differences in spectroscopic behavior of the various chromophore-RBP complexes upon changes in ionic strength and interaction with prealbumin.
absorption maximum, and exhibit optical activity of the chromophore absorption band. Moreover, while apo-RBP is completely dissociated from prealbumin in buffers of physiological ionic strength, the reconstituted retinol isomers-RBP are tightly bound to prealbumin under the same conditions. In addition, the retinol isomer-RBP complexes show a hyperchromic effect of the chromophore absorption band when salt is added to a low ionic strength buffer in the presence of prealbumin. A comparison of the retinol isomers-RBP complexes has shown that the native retinol-RBP complex isolated from human plasma is the all-trans isomer (1).

Goodman and Rae (2) recently have shown that apo-RBP can also combine with retinals and retinoic acid. This paper reports on the spectroscopic properties of reconstituted retinol-RBP, retinyl acetate-RBP, and retinoic acid-RBP. We also report on the interaction of these complexes with prealbumin.

EXPERIMENTAL PROCEDURE

Materials—The preparation of retinol-RBP, prealbumin, and apo-RBP from human plasma was described in the preceding papers (1, 3). All-trans-, 9-cis-, and 13-cis-retinal, all-trans-retinonic acid, and all-trans-retinyl acetate were obtained from Sigma. 11-cis-Retinal was a gift from Hoffman-LaRoche. Emulphogene BC-720 was purchased from General Aniline and Film Co.

Preparation of All-trans-retinol—Preparation was described in the preceding paper (1).

Incubation of Retinals, Retinyl Acetate, and Retinoic Acid with Apo-RBP—Incubations were performed as described in the preceding paper for incubation of retinols with apo-RBP (1).

Binding of Reconstituted Retinol-binding Protein to Prealbumin—Binding studies were performed by gel filtration chromatography with Sephadex G-100 in 0.033 M phosphate buffer, pH 7, containing 0.1 M NaCl as described in the preceding paper (1).

Spectroscopic Studies—Absorption, fluorescence, and circular dichroism spectra were measured as described in the preceding paper (1). Photoselction and measurement of linear dichroism of retinol isomers at 77 K was performed exactly as described in Reference 4.

Protein Concentrations—Protein concentrations were determined from the absorption at 280 nm using an εmax value of 48,000 for retinol-RBP (1, 5), 40,400 for apo-RBP (1), and 78,300 for prealbumin (6, 7).

Retinals, Retinoic Acid, and Retinyl Acetate Concentrations—Concentrations were determined from the absorption at the peak of the long wave length absorption band. The molar absorptivity of all-trans-retinol in ethanol was taken as 42,880; 9-cis-retinol, 36,070; 11-cis-retinol, 24,930; and 13-cis-retinol, 35,500 (8). The molar absorptivity of all-trans-retinyl acetate was taken as 51,500 and that of all-trans-retinoic acid as 45,000 (8).

Nomenclature—As in the case of retinol-RBP complexes, the reconstituted retinals, retinyl acetate, and retinoic acid are explicitly named. The reconstitution of all-trans-retinyl acetate with apo-RBP leads to the formation of all-trans-retinyl acetate-RBP complex.

RESULTS

Reconstitution of Apo-RBP with Retinal Isomers and Retinonic Acid—Similar to the findings of Goodman and Rae (2) we also found that apo-RBP combines not only with retinals, but also with several retinal isomers and with retinoic acid (Table I). Similarly to retinal isomers, all the retinal isomers combined with apo-RBP to form a 1:1 molar ratio complex. We have prepared retinal-RBP complexes several times with different preparations of apo-RBP. In all cases the percentage reconstitution was about the same as reported in Table I, the extreme variations being between about 85 and 100%. When the incubation of retinal with apo-RBP was performed at 4°C, even after 18 hours only about 50% reconstitution took place, whereas at 23°C the process was complete in less than 1 hour. Retinoic acid and retinyl acetate gave a somewhat lower percentage reconstitution (Table I). It is not clear whether this lower percentage reconstitution was real or whether it was due to uncertainties in the molar absorptivity values for these compounds (see below, "Effects of pH on the Absorption and CD Spectra of Retinoic Acid-RBP Complex").

The combination of retinal isomers with apo-RBP led in all cases to a considerable red shift in the absorption peak of the chromophore, ranging from 9 to 12 nm (as compared to the absorption of free retinal in ethanol, Table I). Most interestingly, the recombination of all-trans-retinoic acid with retinol-binding protein shifted its absorption peak to the blue some 15 nm (at pH 9). Similarly to the retinals, the retinol ester retinyl acetate showed a 5-nm shift to the red of the chromophore absorption maximum.

Fluorescence of Retinals and Retinoic Acid-RBP Complexes—Retinal isomers are not fluorescent at room temperature. It was not surprising therefore to find that the various retinol-RBP complexes were nonfluorescent when excited at 350 and 450 nm and measured between 380 and 600 nm. The only indication that a complex between retinal and RBP was formed was the marked quenching of the protein fluorescence (Table II). A control experiment showed that the addition of free retinal to retinol-RBP complex did not cause any quenching. When all-

| Compound | Free chromophore | Reconstituted retinal, retinyl acetate, or retinoic acid-RBP complex | |
|----------|-----------------|---------------------------------------------------------------------|---|
|          | max nm          | max nm                                                              | per cent | shift of absorption |
| all-trans retinal | 362 | 1.12 | 294 | red, 11 |
| 13-cis retinal | 375 | 1.40 | 384 | red, 9 |
| 11-cis retinal | 372 | 1.05 | 491 | red, 12 |
| apo-RBP | 372 | 1.05 | 491 | red, 12 |
| all-trans retinyl acetate | 372 | 1.15 | 428 | red, 9 |
| all-trans retinoic acid | 370 | 0.96 | 355 | blue, 15 |
| all-trans retinyl acetate | 372 | 1.14 | 420 | red, 15 |

* Values, in ethanol, are taken from Reference 7.

b Assuming a 1:1 molar ratio of protein to chromophore as 100%.

At pH 9.
Apo-RBP, in 3 ml of 0.033 m sodium phosphate buffer, pH 7.0, containing 0.1 m NaCl, was incubated with retinals, retinoic acid, or all-trans-retinol added in 5 μl of ethanol. The chromophore solution (1 mg per ml) was always freshly made. Readings were taken after incubation in the dark for 30 min at 23°C. All operations were carried out under dim red light.

| excitation wavelength, nm | emission wavelength, nm | Fluorescence, arbitrary units |
|---------------------------|-------------------------|------------------------------|
|                           |                        | apo-RBP                      |
|                           | all-trans retinal       |                             |
|                           | 13-cis retinal         |                             |
|                           | 11-cis retinal         |                             |
|                           | 9-cis retinal          |                             |
|                           | all-trans retinoic acid |                             |
|                           | all-trans retinal       |                             |
| 380                       | 470                    | 7                            |
| 280                       | 470                    | 8                            |
| 280                       | 330                    | 5                            |

* First addition to the apo-RBP.

+ After the first incubation, all-trans-retinol was added and the material was incubated for a further 30 min at 23°C.

The excitation wavelength was 330 nm.

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**Fig. 1.** CD spectra of retinal isomers-RBP. All compounds (final concentration, 30 μM) were dissolved in 2 mM Tris-HCl, pH 9.0. The path length was 10 mm. Each spectrum represents the average of 16 scans.

*trans*-retinol was added to a retinal-RBP complex there was very little increase in fluorescence (Table IV, below). This was interpreted to mean that the retinals occupied the same site as the retinols in retinol-binding protein and that this site was already fully saturated by retinals. Similar results were also obtained with all-trans-retinoic acid.

**Circular Dichroism of Retinal Isomers-, Retinyl Acetate-, and Retinoic Acid-RBP Complexes**—Whereas free retinols and retinals in solution are devoid of optical activity, retinol isomers-RBP complexes show an extrinsic Cotton effect of the chromophore absorption band (1). Similarly, the retinal isomers-RBP complexes show a CD band at their chromophore absorption band (Fig. 1). The CD bands of the retinal-RBP complexes peak at about the same wavelength as the absorption peak of the complex (compare Table I and Fig. 1). The rotatory strength of the retinal-RBP complexes was about the same as that of the retinol-RBP complexes (Table III; compare Table I in Reference 1). The all-trans-retinyl-RBP complex possessed the largest CD band similar to that of the all-trans isomer among retinol-RBP complexes.

Both the all-trans-retinyl acetate- and the all-trans-retinoic acid-RBP showed an induced CD band, peaking at about the same wavelength as the chromophore absorption band and again showing a rotatory strength similar to that of the retinol- and retinal-RBP complexes (Table III).

In a previous paper (3) we have shown that all-trans-retinol combines with bovine serum albumin to give a relatively stable complex that can be recognized by gel filtration chromatography and by enhancement of the retinol fluorescence quantum yield. All-trans-retinal also combines with bovine serum albumin to give a complex with an A₂₆₀/A₄₅₀ ratio of 0.25, after gel filtration chromatography to remove excess retinal. Neither the all-trans-retinol nor the all-trans-retinyl-bovine serum albumin complex showed any optical activity of the bound chromophore absorption band. At the level of sensitivity used for the CD measurements both the all-trans-retinol- and the retinyl-bovine serum albumin complexes had an induced optical activity band at least 100 times smaller than that of the corresponding retinol-binding protein complex—if they had any optical activity at all. We interpreted these findings to show that the induced optical activity of the retinol-, retinyl-, and retinoic acid-RBP complexes was not merely an expression of binding, as such, but of a highly specific binding which results in an asymmetric chromophore-protein complex.

**Effects of pH on the Absorption and CD Spectra of Retinoic Acid-RBP**—To investigate the possible effect of pH on the absorption and CD spectra of retinoic acid-RBP, the following experiments were performed:

1. **Absorption Spectra**—The absorption spectra of retinoic acid-RBP were recorded at different pH values (2.5–10.0) using spectroscopy-grade materials and 0.1 M NaOH and 0.1 M HCl as buffers. The CD spectra of retinoic acid-RBP complexes were recorded at 3.0 ± 0.5 pH units from 300 to 450 nm.

2. **Circular Dichroism Spectra**—The CD spectra of retinoic acid-RBP complexes were recorded at different pH values (2.5–10.0) using spectroscopy-grade materials and 0.1 M NaOH and 0.1 M HCl as buffers. The CD spectra were recorded at 3.0 ± 0.5 pH units from 300 to 450 nm.

3. **Circular Dichroism Measurements**—The CD measurements were performed using a JASCO J-810 spectropolarimeter equipped with a 1-cm path-length cell. The CD spectra were recorded at room temperature (25°C) and at pH values ranging from 2.5 to 10.0. The CD spectra were recorded at 3.0 ± 0.5 pH units from 300 to 450 nm.

**Table III**

| Compound                     | Rₛ | Dobyle-Bohr magnetons |
|------------------------------|----|-----------------------|
| all-trans retinyl acetate-RBP | 0.77 | 0.77                  |
| all-trans retinoic acid-RBP  | 0.77 | 0.77                  |
| all-trans retinal-RBP        | 0.77 | 0.77                  |
| 13-cis retinal-RBP           | 0.56 | 0.43                  |
| 11-cis retinal-RBP           | 0.39 | 0.22                  |
| 9-cis retinal-RBP            | 0.29 | 0.09                  |
| all-trans retinyl acetate-RBP| 0.77 | 0.77                  |
| all-trans retinoic acid-RBP  | 0.77 | 0.77                  |
Acid-RBP Complex—When free all-trans-retinoic acid was dissolved in aqueous 1% Emulphogene (a nonionic detergent) both the peak height and the peak position were pH dependent (Fig. 2A). Thus, while the peak position at pH 3.5 was at 355 nm, the peak position at pH 9.3 was at 341 nm and there was a concomitant 17% increase in peak height. There was no change in area under the chromophore absorption band (270 to 440 nm) upon changes in pH. It is clear that the ionization of the carboxyl group of retinoic acid has a marked effect on both the peak position and the peak height, the charged form having a higher peak and showing a blue shift.

When the same series of experiments was performed with an all-trans-retinoic acid-RBP complex, similar changes were observed. The chromophore peak position shifted from about 335 nm at pH 9 and pH 8 to about 355 nm at pH 3.65 (Fig. 2B). There was a concomitant decrease in peak height, with the peak at pH 8 being 43% higher than that at pH 3.65. There was a small decrease in peak height at 280 nm in going from pH 9 to pH 8, with only minor changes upon further lowering of the pH (Fig. 2B). These changes in the chromophore absorption were partially reversible. When the pH was brought back from 3.65 to 9, the peak position shifted back to 335 nm, but there was a loss of about 12% in intensity, probably due to destruction of the chromophore-RBP complex at acid pH (Fig. 2B, Curve 6). Supporting this interpretation was a 10% decrease in the size of the chromophore absorption band (290 to 450 nm) in going from pH 9 to pH 3.65, and a further 6% decrease when the pH was brought back to 9.0. The same reversible changes in the absorption spectrum of retinoic acid-RBP complex as a function of pH were seen in low and physiological ionic strength buffers.

Changes in pH also produced a shift in the CD spectrum of all-trans-retinoic acid-RBP. The peak position of the CD spectrum shifted from about 330 nm at pH 9 and pH 8 to about 340 nm at pH 3.65 (Fig. 3), with a concomitant 14% decrease in rotatory strength. The changes in the CD spectrum were also reversible, with the peak position shifting back to about 330 nm in going from pH 3.65 to pH 9.0 and a further 6% decrease in rotatory strength. Like the decrease in area under the absorption band, the decrease in rotatory strength was probably due to progressive destruction of the retinoic acid-RBP complex at acid pH. The changes in the CD spectrum of retinoic acid-RBP complex as a function of pH were the same in low and physiological ionic strength buffers.

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**TABLE IV**

**Effect of ionic strength on the absorption and circular dichroism spectra of retinal isomers- and retinoic acid-RBP complexes**

| RBP complex | all-trans retinal | 9-cis retinal | 11-cis retinal | 13-cis retinal | all-trans retinoic acid |
|-------------|------------------|--------------|---------------|---------------|-------------------------|
| Absorption spectrum |
| λ max, nm (low ionic strength) | 394 | 383 | 391 | 364 | 334 |
| λ max, nm (physiological ionic strength) | 393 | 377 | 391 | 383 | 335 |
| change in absorption in going from low to physiological ionic strength | -2 | -2 | 0 | +2.4 |
| Circular dichroism |
| λ max, nm (low ionic strength) | 395 | 380 | 390 | 380 | 335 |
| λ max, nm (physiological ionic strength) | 393 | 379 | 390 | 379 | 335 |
| change in CD in going from low to physiological ionic strength | -2 | -2 | -2 | -1 |

* In 2 mM Tris-HCl, pH 9.
* In 2 mM Tris-HCl, pH 9, containing 0.15 M NaCl.
* Calculated as the change in area under the absorption or CD band between 300 and 600 nm. The area at low ionic strength was taken as 100%.
* Broad CD bands.

**Fig. 2.** Effect of pH on the absorption spectra of retinoic acid and retinoic acid-RBP complex. A, all-trans-retinoic acid in 1% Emulphogene-2 mM Tris. The pH was adjusted by adding 1 or 2 μl of either 5 M or 1 M NaOH or HCl, and measured with a glass electrode and a Radiometer pH meter. The path length was 10 mm. The pH values were: 1, pH 9.3; 2, pH 7.7; 3, pH 6.45; 4, pH 5.4; and 5, pH 3.5. B, all-trans-retinoic acid-RBP complex (after chromatography on Sephadex G-25) in 2 mM Tris. The pH was adjusted and measured as in A. The path length was 10 mm. The pH values were: 1, pH 8.0; 2, pH 9.0; 3, pH 7.0; 4, pH 5.95; and 5, pH 3.65; ε was measured after the pH was brought back to 9.0.

**Fig. 3.** Effect of pH on the CD spectrum of retinoic acid-RBP complex. The sample was the same as that shown in Fig. 2B. The path length was 10 mm. Each curve represents the average of eight scans. The pH values were: 1, pH 8.0 and 9.0; 2, pH 7.0; 3, pH 5.95; and 4, pH 3.65; ε was determined after the pH was brought back to 9.0.
Possible Covalent Linkage of Retinal to Retinol-binding Protein—

Unlike retinols, which are fairly unreactive alcohols, the reactive aldehyde function of retinal has the potential to react with several reactive sites on the protein, most probably with a primary amino group. It should be recalled in this connection that in the analogous situation of visual pigments, the retinal chromophore is covalently linked to an ε-amino group of a lysine residue in rhodopsin.

Two methods were used to establish whether retinal was present as the free aldehyde or was covalently linked as a Schiff base.

Reduction of the free aldehyde with sodium borohydride produces the corresponding alcohol, while reduction of the Schiff base leads to a new covalent linkage between retinal and the amino group in the form of a stable primary amino linkage (9). Reduction of all-trans-retinal-RBP with NaBH₄ produced a species that had an absorption spectrum similar to that of all-trans-retinol-RBP and which showed optical activity of the chromophore absorption band. One extraction of this reduced compound with ethanol (1) removed about 80% of the chromophore as free retinol. This was approximately the same amount of retinol that could be extracted from native retinol-RBP by one ethanol extraction (1). The facts that the reduced all-trans-retinal in retinol-binding protein could be extracted with ethanol and that it had absorption and CD spectra similar to that of native retinol-RBP were taken as evidence that retinal in retinol-binding protein could be extracted with ethanol and that the binding site of retinol-binding protein resembled more closely the hydrophobic environment of digitonin micelles than that of the more hydrophilic environment of the ethanol-glycerol solution.

The second approach was to use the technique of photoselection and linear dichroism that was recently developed for identification and quantitative measurement of retinal isomers (4). The LD spectra of retinal isomers are unique and different from those of Schiff bases and other compounds of retinal (10). Retinols do not show photoselection and linear dichroism (10).

Using this technique, the retinal isomers-RBP gave LD spectra similar to that of the corresponding free retinal (Fig. 4). Although the general shape of the LD spectrum of each retinal isomer was unique, the fact that the LD spectra were different from those of Schiff bases is in itself evidence that the retinals were dissolved in ethanol-glycerol (1:1) v/v and that the free aldehyde function can be reduced in situ to produce the corresponding retinol-RBP complex.

We interpret these findings as showing that the retinal in retinol-binding protein was present in the free aldehyde form and that the binding site of retinol-binding protein resembled more closely the hydrophobic environment of digitonin micelles than that of the more hydrophilic environment of the ethanol-glycerol solution.

Since under standardized conditions the magnitude of the LD signals of retinal isomers is linearly related to concentration, it is possible to correlate these two measurements (4). The magnitude of the LD signals, which were obtained from a given amount of retinal-RBP complex (as determined by absorption spectroscopy) was the same as that obtained from the equivalent concentration of free retinal. We interpreted this finding to mean that all the retinal complement of a retinal-RBP complex is in the free aldehyde form, because any other linkage would give a smaller LD signal for a given concentration.

Effects on Absorption and CD Spectra of Increase in Ionic Strength—When solid NaCl (final concentration 0.15 M) was added to solutions of retinal- and retinoic acid-RBP complexes in 2 mM Tris buffer, pH 9, changes were observed in both the absorption and the CD spectra (Table IV). In no case was there a change in the absorption spectrum at the 280-nm band (Fig. 5). In all cases, though, there was an effect on the chromophore absorption band, which was shifted to the blue, 1 to 7 nm, in the case of the retinal-RBP complexes, and about 1 nm to the red in the retinoic acid-RBP complex (Table IV and Fig. 5). Although the absorption spectrum did shift considerably, as for instance in the case of the 9-cis-retinal-RBP, the area under the absorption curve decreased only by some 2.7% as compared to that in low ionic strength.

Accompanying the change in the absorption spectrum, there were also changes in the CD spectrum. The peak of the CD band also shifted to the blue upon increase in ionic strength. The decrease in rotatory strength was considerable, amounting to some 39% in 11-cis-retinal-RBP complex (Table IV and Fig. 6). The all-trans-retinoic acid-RBP showed a small increase in rotatory strength. There were also changes in the far ultraviolet CD spectra (200 to 240 nm) of the retinal- and retinoic acid-RBP complexes. These changes were on the order of up to 10% in peak height, with small shifts in peak position (on the order of 2 nm).

When the free retinal isomers were dissolved in 1% Emulphogene BC-720 in 2 mM Tris, pH 9, and solid NaCl was added
FIG. 5. Effect of ionic strength on the absorption spectrum of 9-cis-retinal-RBP. The absorption spectrum of 9-cis-retinal-RBP in 2 mM Tris, pH 9.0, was recorded (---), and solid NaCl was then added to a final concentration of 0.15 M (---).

![Figure 5](image)

FIG. 7. Absorption spectrum of 9-cis-retinal-RBP in the absence or presence of prealbumin. 1, absorption spectrum of 9-cis-retinal-RBP complex (16.6 μM) in 1.4 ml of 2 mM Tris, pH 9.0, after gel filtration chromatography on Sephadex G-25. 2, prealbumin, 23.4 nmoles in 0.29 ml of 2 mM Tris, pH 9.0, was added to 1 above. 3, solid NaCl (final concentration, 0.15 M) was added to 2. Absorptions of 2 and 3 are +1 between 265 and 293 nm.

![Figure 7](image)

Interaction between Retinal-, Retinyl Acetate-, and Retinoic Acid-RBP Complexes and Prealbumin—In contrast to the retinal-RBP complexes, all of which were bound to prealbumin at physiological ionic strength, none of the retinal RBP complexes were bound to prealbumin under the same conditions, as judged by gel filtration chromatography. All the retinal-RBP complexes appeared from the gel filtration column as separate peaks at the same position as that of apo-RBP and retinol-RBP in the absence of prealbumin. Although the retinal-RBP complexes were completely dissociated from prealbumin, they still had all the retinal bound to retinol-binding protein. Most interestingly, while all-trans-retinol-RBP was bound to prealbumin at physiological ionic strength, the acetate ester, namely, all-trans-retinyl acetate-RBP, was completely dissociated. On the other hand all-trans-retinoic acid-RBP was bound firmly to prealbumin and appeared from the gel filtration column as one peak at the same position as that of retinol-RBP-prealbumin complexes.

Although the binding studies using the gel filtration technique showed that there was no binding between retinal-RBP and prealbumin, the interactions between the proteins were quite complex as judged by absorption and CD spectroscopy. Addition of prealbumin to a solution of retinal isomers-RBP at low ionic strength shifted the absorption maximum to the blue some 8 nm with 9-cis-retinal-RBP and 5 nm with 11-cis-retinal-RBP (Table IV and Fig. 7). There were no apparent shifts with all-trans-retinal-, 13-cis-retinal-, and retinoic acid-RBP complexes.

When solid NaCl was added to solutions containing retinal-RBP and prealbumin at low ionic strength there was a further shift to the blue with all the retinal-RBP complexes and a small shift to the red with retinoic acid-RBP. In all these cases, namely, both at low and high ionic strength, there was no change at all in the absorption at 280 nm.

It is interesting to note that whereas increasing the ionic strength of a retinal-RBP solution alone led either to a small decrease in the chromophore absorption band or to no change, an increase in the ionic strength of a mixture of retinal-RBP and prealbumin led to an increase in the chromophore absorption band (Tables IV and V).

The analogous situation was seen with retinoic acid-RBP. There was an increase of some 2.4% in the chromophore absorption when salt was added to retinoic acid-RBP alone (Table IV) and a further 2.3% increase, to a total of 4.7%, when salt was added to a retinoic acid-RBP and prealbumin mixture.
The retinal visual pigment (rhodopsin) and the complexes all have a CD band in the 320- to 340-nm region (Fig. 1). The retinal visual pigment (isorhodopsin) also show a prominent Cotton effect at about the same region—the so-called β band (11). The CD bands of the retinals-RBP and the visual pigments peak at about the same wave length, and they are quite strong despite the fact that there is no clear absorption band in this region in the retinal-RBP complexes (Fig. 7). It is an attractive hypothesis that the CD bands at about 320 to 340 nm in both the retinal-RBP complexes and visual pigments are derived from the same inherent optical transition of the retinal chromophore.

Retinals possess a reactive aldehyde function that under the proper conditions reacts rapidly with primary amino groups to give Schiff bases. It is interesting that even when large excesses of retinal were incubated with retinol-binding protein none combined covalently with the protein. It is well known that most primary amino groups of proteins (ε-amino of lysine residues) are "surface" groups and thus would be expected to be readily available. We do not have an explanation at this time for the marked lack of reactivity of retinals toward the primary amino groups of retinol-binding protein. We also have observed that retinals do not react readily with the amino groups of the visual pigment apoprotein (opsin) other than the group involved in the usual chromophore binding.

As would be expected, the spectrum of free retinoic acid showed a marked dependence on pH. It was somewhat surprising to find that the absorption spectrum of retinoic acid-RBP was also pH dependent and that spectral changes due to pH change were reversible. That this was not due to denaturation and release of free retinoic acid from retinol-binding protein was evident from the CD spectrum which also was reversibly pH dependent. Thus we are led to conclude that the carboxyl group of retinoic acid-RBP complex is available to the solvent. We have shown also that the aldehyde function of retinal-RBP can be reduced in situ with NaB₃H₄ to retinol-RBP, apparently without destruction of the complex. The availability for interaction with solute of both the carboxyl and aldehyde groups of retinoic acid- and retinal-RBP complexes was distinct from that of retinol-RBP, in which the alcohol seems to be unavailable and protected from the solvent (3, 5).

Although retinals, retinols, retinoic acid, and retinyl acetate all bind to the same site on retinol-binding protein, the resulting conjugated chromophore-proteins differ in some notable ways. The most striking is the difference in binding to prealbumin at physiological ionic strength. Only the retinol- and retinoic acid-RBP complexes were bound. This striking difference could be due either to a direct interference in the binding between prealbumin and retinol-binding protein by the functional group of the polychrome chromophore or to variation in conformation induced in retinol-binding protein by the different chromophores. Because of the experimental evidence showing that retinol is highly protected from interactions with various reagents when it is bound to retinol-binding protein, the retinyl function would not. It seems easier to assume that the different functional groups impart a somewhat different conformation to the reconstituted retinol-binding protein and that this in turn impairs the binding between the two proteins.

A somewhat similar explanation for the effect of increased ionic strength on the absorption of retinal- and retinoic acid-RBP (Table IV) seems reasonable. Changes in ionic strength had absolutely no effect on the spectra of free retinals dissolved in an aqueous nonionic detergent solution. The ionic strength effect was mediated then through electrostatic effects on the tertiary structure of the protein, which affected in turn the bind-

| Table V |
| --- |
| Interaction of prealbumin with retinal- and retinoic acid-RBP at low and physiological ionic strengths |
| | all-trans retinal | 9-cis retinal | 11-cis retinal | 13-cis retinal | all-trans retinoic acid |
| | | | | | |
| +PA | 1 max, nm | 394 | 375 | 386 | 384 |
| low ionic strength | | 373 | 366 | 374 | 335 |
| -PA | 1 max, nm | 394 | 386 | 391 | 384 |
| physiological | 394 | 370 | 354 | 377 | 336 |
| change in | absorbance in | +1.2 | +1.0 | +2.5 | +2.3 |
| ionic strength | going from | 370 | 366 | 379 | 336 |
| per cent | low to | +4.7 |

a The abbreviation used is: PA, prealbumin. Low ionic strength solutions were 2 mM Tris-HCl, pH 9.

b In 2 mM Tris-HCl, pH 9, containing 0.15 M NaCl.

c Calculated as the area in change under the absorption band between 300 and 600 nm. The area at low ionic strength was taken as 100%.

Discussion

The results reported in this paper further elaborate on the theme of the relatively low specificity as to nature and geometrical isomerism of the chromophores that bind to apo-RBP. The analogs of all-trans-retinal, namely the alcohols (retinol isomers), aldehydes (retinals), acids (retinoic acid), and esters (retinyl acetate) bind with about the same high effectiveness to apo-RBP; all seem to be bound at the same site on retinol-binding protein and are thus competitive inhibitors. This situation of low selectivity in the nature of the chromophore which is bound to retinol-binding protein stands in marked contrast to the low selectivity with apo-RBP; all seem to be bound at the same site on retinol-binding protein and are thus competitive inhibitors. This hypothesis that the CD bands at about 320 to 340 nm implicate direct interference by the polyene functional group in the binding of prealbumin to retinol-binding protein. Xoreover, it is not obvious from this hypothesis why the aldehyde function of retinoic acid-RBP complex is available to the solvent. We have shown also that the aldehyde function of retinal-RBP can be reduced in situ with NaB₃H₄ to retinol-RBP, apparently without destruction of the complex. The availability for interaction with solute of both the carboxyl and aldehyde groups of retinoic acid- and retinal-RBP complexes was distinct from that of retinol-RBP, in which the alcohol seems to be unavailable and protected from the solvent (3, 5).

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ing site and interactions between retinals and retinol-binding protein. The marked changes in the induced Cotton effect of the chromophore absorption band (Table IV) can be taken as further support for this hypothesis.

It is interesting that although retinal-RBP complexes do not bind to prealbumin as judged by gel filtration chromatography, there were interactions between the proteins even at low ionic strength. The addition of prealbumin to 9- and 11-cis-retinal-RBP at low ionic strength caused a shift of the chromophore absorption to the blue. Increasing the ionic strength caused a further shift to the blue (Table V). It is clear that the two proteins interacted even in low ionic strength buffer, although firm binding was not detected. Recall that prealbumin did not change the chromophore peak position of retinol isomers-RBP either at low or at physiological ionic strength, although there was a hyperchromic effect on the absorption band when salt was added (1).

A more complete understanding of all these fascinating effects on the absorption, CD, and binding properties of the various chromophore-RBP complexes will have to await a better knowledge of the structural aspects of the polyene chromophore-binding site of retinol-binding protein.

Acknowledgments—We are deeply indebted to Mrs. Victoria Wong and Mrs. Marianne Lawrence for their excellent technical help. It is a pleasure to thank Beckman Instruments for their generosity in providing us with the use of the circular dichroism spectrophotometer. We thank Dr. E. H. Strickland for making his fluorometer available to us.

REFERENCES

1. Heller, J., and Horwitz, J. (1973) J. Biol. Chem. 248, 6308-6316
2. Goodman, D. W., and Raz, A. (1972) J. Lipid Res. 13, 338-347
3. Futterman, S., and Heller, J. (1972) J. Biol. Chem. 247, 5168-5172
4. Horwitz, J., and Heller, J. (1973) J. Biol. Chem. 248, 1051-1055
5. Kanai, M., Raz, A., and Goodman, D. W. (1968) J. Clin. Invest. 47, 2025-2044
6. Raz, A., and Goodman, D. W. (1969) J. Biol. Chem. 244, 3220-3227
7. Branch, W. T., Jr., Robbins, J., and Edelhoch, H. (1971) J. Biol. Chem. 246, 6011-6018
8. Hubbard, R., Brown, P. K., and Bownds, D. (1971) Methods Enzymol. 18C, 628-629
9. Fischer, E. H. (1965) in Structure and Activity of Enzymes (Goodman, T. W., Harris, J. I., and Hartley, B. S., eds) pp. 111-120, Academic Press, New York
10. Heller, J., and Horwitz, J. (1973) in Biochemistry and Physiology of Visual Pigments (Langer, J., ed) pp. 57-68, Springer-Verlag, Berlin
11. Shaw, T. I. (1972) in Handbook of Sensory Physiology (Dartnall, H. J. A., ed) Vol. VII/1, pp. 180-199, Springer-Verlag, Berlin
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J. Biol. Chem. 1973, 248:6317-6324.

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