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The Interaction of Human Plasma Retinol-binding Protein with Prealbumin*

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SUMMARY

The interaction of human plasma retinol-binding protein with plasma prealbumin was studied by the techniques of velocity ultracentrifugation and polarization of retinol fluorescence. In the first method the unbound fraction of retinol-binding protein, which sediments more slowly than its complexes with prealbumin, was measured by its absorption. A stoichiometry of retinol-binding protein to prealbumin, 4:1 was found. The polarization of fluorescence of retinol increases when retinol-binding protein combines with prealbumin; the relaxation time of retinol-binding protein was 6.6 $\times$ 10^{-8} s at 25° and increased to 28 $\times$ 10^{-8} s for the complex. The polarization data indicate that there are four independent binding sites with an apparent association constant of approximately 1.2 $\times$ 10^{6} M^{-1} at 25° and pH 7.4. The affinity was maximal near pH 7.4 and decreased gradually at lower and higher pH values. Between pH 6.0 and 9.4, the extent of interaction between retinol-binding protein and prealbumin was dependent on ionic strength. In this pH range almost no binding occurred without added salt and the affinity increased rapidly with increasing KCl concentration to 0.1 M. At pH values below 6 the interaction was less dependent on ionic strength and became independent at pH 4.2.

Vitamin A is transported by a specific serum protein which is known as retinol-binding protein (RBP). This protein was first isolated by Kanai *et al.* (1) and has been studied extensively (1–13). It has an α_1 electrophoretic mobility and a molecular weight of about 21,000, and it binds 1 mole of retinol per mole of protein. A very similar transport protein for retinol is found in the rat (14). The secretion of this protein by the liver is apparently blocked when retinol is not available (15). The association of RBP[1] with prealbumin (1, 3), which has a molecular weight of about 54,000 (16), prevents its filtration through the glomeruli.

Prealbumin is one of three serum proteins responsible for thyroxine transport (17, 18). It is a very stable tetramer of four identical subunits (4, 16, 19–24) with a single, strong thyroxine-binding site (2, 25, 26), although several weaker sites may exist (26). X-ray analysis of the three-dimensional structure of prealbumin has revealed a tetrahedral arrangement of the subunits with a channel through the center of the molecule (22, 23). This space may be the site of thyroxine binding.

Studies on RBP binding indicated a single binding site on prealbumin which is independent of the thyroxine site (1, 3, 10). We have re-evaluated the binding of retinol-binding protein to prealbumin by ultracentrifugation and polarization of fluorescense. The data suggest that there are four independent RBP-binding sites on prealbumin. The effect of pH and ionic strength on the interaction has also been ascertained.

EXPERIMENTAL PROCEDURE

Retinol-binding Protein and Prealbumin Preparations

Retinol-binding protein was isolated from outdated human plasma by the procedures previously described (1–3). The purified material was lyophilized and kept at −20° until used. Lyophilized human prealbumin was purchased from Behring Werke, Marburg Lahn, Germany, and further purified by preparative polyacrylamide electrophoresis as described elsewhere (16).

Protein Concentration

Protein concentration was measured by absorption at 280 nm. $E^{1\%}_{1cm}$ = 14.1 was used for prealbumin (2). The extinction coefficient of the retinol-binding protein preparation was determined as follows. Retinol-binding protein was dialyzed thoroughly against 0.1 M KCl + 0.05 M phosphate buffer, pH 7.4. The concentration was then measured with a Brice-Phoenix differential refractometer and also by micro-Kjeldahl analysis for nitrogen. A nitrogen content of 16.94% was computed

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[1] The abbreviations used are: RBP, retinol-binding protein; holo-RBP, RBP containing retinol; apo-RBP, RBP devoid of retinol.

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from the amino acid composition of retinol-binding protein (1, 5). Both methods gave an $E^{1\%}_{1cm}$ at 280 nm of 16.8. The absorbance ratio (330:280 nm) of the retinol-binding protein solution was 0.79.

Ultracentrifugation

Sucrose density gradient centrifugation was performed at 20° in linear 5 to 40% gradients with the SW 41 rotor of the Spinco model L2-65B ultracentrifuge. Solutions were centrifuged at 40,000 rpm for 48 hours. The tubes were sampled by collecting drops through a needle from the bottom of the centrifuge tube.

Sedimentation velocity was performed with the Spinco model E ultracentrifuge equipped with a photoelectric ultraviolet scanner. Double sector cells were used in the AnG rotor at 48,000 rpm and the temperature was controlled at 20° by using an RTIC unit (Arden Instruments, Inc., Rockville, Md.).

Fluorescence

Relative protein and retinol concentrations of sucrose gradient fractions were determined by measuring the fluorescence of solutions in RTU tubes (10 × 75 mm) (Becton, Dickinson & Co., Rutherford, N. J.) with an Amico Bowman spectrofluorometer. Fluorescence spectra were obtained with a Turner model 210 spectrofluorometer. Excitation and emission wave lengths were at 295 and 345 nm for tryptophan and at 330 and 480 nm for retinol. The absorbance of all solutions was less than 0.08 at the excitation wave length.

Polarization of Fluorescence

The Phoenix light-scattering photometer was used to measure polarization of fluorescence of retinol-binding protein and RBP-prealbumin complexes. Corning filters 7-54 and 3387 were used to intercept the incident (unpolarized) and fluorescent beams, respectively, in order to obtain excitation and emission wave lengths of 330 nm and 480 nm. The temperature was controlled by circulating water from a constant temperature bath through a water-jacketed cell holder. The intensities of the vertical (I_V) and horizontal (I_H) components of the fluorescent light were measured at an angle of 90° to the incident beam. Polarization (P) is then defined as $(I_V - I_H)/(I_V + I_H)$. The relaxation time (ρ_h) is related to the temperature (T) and viscosity (η) of the medium according to the Perrin equation:

$$\left(\frac{1}{P} + \frac{1}{3}\right) = \left(\frac{1}{P_0} + \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \qquad (1)$$

where P_0 is the limiting value of P when T/η is zero and τ is the fluorescence lifetime. ρ_h is calculated from the slope and intercept of a plot of $1/P + 1/3$ *versus* T/η, *i.e.* $\rho_h^{25°}$ = intercept/slope $3\tau\ (\eta/T)^{25°}_{H_2O}$. The relaxation time ($\rho_0$) of a rigid, unhydrated sphere of the same mass can be calculated as $\rho_0^{25°} = 3\ V\eta/RT$, where $V = M\bar{v}$; M is the molecular weight and $\bar{v}$ is the partial specific volume.

Fluorescence Lifetime

Fluorescence lifetime was determined with a modified nanosecond decay time apparatus (TRW, Inc., El Segundo, Calif.) by the method described by Chen (27).

Binding Experiments

Sedimentation Velocity—Solutions of known retinol-binding protein and prealbumin concentrations were centrifuged and the absorption pattern in the cell was measured after the faster sedimenting boundary containing prealbumin and the RBP-prealbumin complexes was resolved from the slower sedimenting free retinol-binding protein boundary (2.3 S). There is very little increase in the sedimentation rate of prealbumin with increasing binding of retinol-binding protein. The absorbance of the free retinol-binding protein was obtained either from the RBP plateau or from the inflection point of the free RBP boundary. When the latter value was doubled both methods gave similar values for free retinol-binding protein. The concentration of prealbumin (C_t) in the plateau region of the RBP-prealbumin complex was taken as the initial concentration of prealbumin (C_0) corrected for radial dilution, *i.e.* $C_t/C_0 = (r_m/r_t)^2$, where r_m and r_t are the radial positions of the meniscus and the boundary of the RBP-prealbumin complex, respectively. The relative concentration of free retinol-binding protein in the presence of prealbumin was determined by comparing the absorbance of the free RBP plateau with that of a known concentration of retinol-binding protein without prealbumin and with appropriate corrections for radial dilution.

Polarization of Fluorescence—The binding of retinol-binding protein to prealbumin was determined from polarization values by titrating RBP solutions with prealbumin. We have assumed that the two limiting polarization values, *i.e.* in the absence of prealbumin and in excess of prealbumin, represent the values for free and bound retinol-binding protein, respectively. The analysis further assumes that the higher complexes have the same polarization values as the 1:1 complex. This appears to be valid since the polarization values do not fall in the presence of a large excess of prealbumin where each prealbumin can bind only one retinol-binding protein. The fraction of bound RBP was obtained from the relation $(P - P_{min})/(P_{max} - P_{min})$. The free retinol-binding protein could then be calculated since the total RBP concentration was known.

Analysis of Binding Data—The binding of retinol-binding protein to prealbumin (PA), PA + nRBP $\rightleftharpoons$ PA·RBPn, can be expressed as $\bar{\nu} = (nKC_{RBP})/(1 + KC_{RBP})$ for a single class of independent binding sites, where $\bar{\nu}$ is the number of moles of retinol-binding protein bound per mole of prealbumin, n is the total number of binding sites on prealbumin, K is the association constant, C_{RBP} is the molar concentration of free retinol-binding protein, and C^T_{RBP} and C^T_{PA} are the total concentrations of retinol-binding protein and prealbumin, respectively. C^B_{RBP} is the concentration of bound retinol-binding protein.

Thus, $C^T_{RBP} = C^B_{RBP} + C_{RBP} = \bar{\nu}C^T_{PA} + C_{RBP} = (nKC_{RBP} \cdot C^T_{PA})/(1 + KC_{RBP}) + C_{RBP}$. The following equation, obtained by rearranging terms, was used to analyze the binding data by curve fitting:

$$KC^2_{RBP} + C_{RBP}\ (1 + nKC^T_{PA} - KC^T_{RBP}) - C^T_{RBP} = 0 \qquad (2)$$

The various programs used for this purpose were developed by Dr. H. Saroff of the National Institutes of Health.

RESULTS

Sedimentation Velocity—The retinol-binding protein preparation employed in this study consisted of a mixture of holo-RBP and apo-RBP. The ratio of the absorbance at 330 to 280 nm affords a measure of the relative amount of holo-RBP present in any preparation. A ratio of 1.05 indicates that all of the protein contains retinol (1). The retinol-binding protein used in this study had a ratio of 0.79.

Since about 25% of the protein was in the apo-form it was necessary to ascertain whether all of the protein in the retinol-

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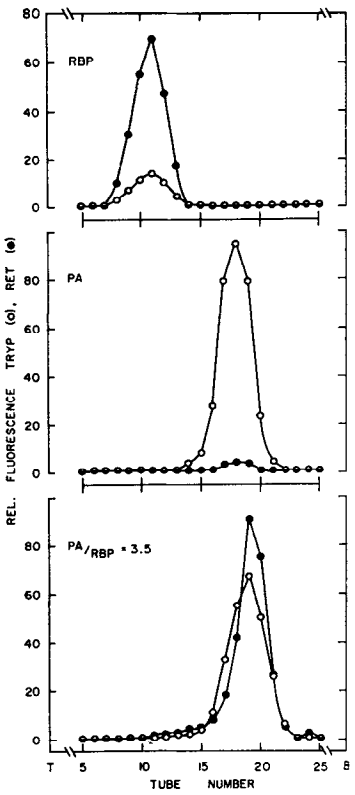

FIG. 1. Density gradient ultracentrifugation of retinol-binding protein (*RBP*) (6.3 μM), prealbumin (*PA*) (22 μM), and a mixture of the two proteins at the same concentrations; 5 to 40% sucrose gradient in 0.05 M phosphate, 0.1 M KCl, pH 7.4; SW 41 rotor; centrifugation at 40,000 rpm for 48 hours. *T* and *B* indicate top and bottom fractions, respectively. Retinol (*ret*) and tryptophanyl (*tryp*) fluorescence were measured at 480 nm (●) and 340 nm (○), respectively. The small amount of 480 nm fluorescence in the *middle panel* is from an unknown non-protein impurity in the prealbumin preparation.

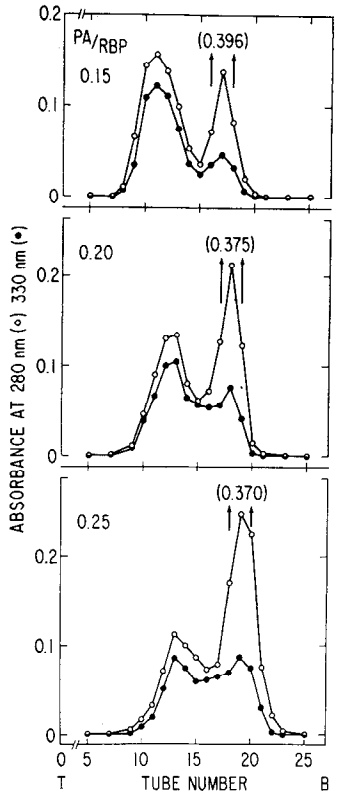

FIG. 2. Density gradient ultracentrifugation of retinol-binding protein (*RBP*) (47 μM) and prealbumin (*PA*) mixtures at the indicated mole ratios. The *numbers in parentheses* are the absorbance ratios (330 to 280 nm) for the average of three fractions from the rapidly sedimenting zone. Other conditions are similar to those in Fig. 1.

binding protein preparation was bound to prealbumin. Therefore, the sedimentation behavior of retinol-binding protein (6.3 μM) in the presence of a large excess of prealbumin (22 μM) was analyzed by sucrose gradient centrifugation. The sedimentation profiles of the individual proteins and the mixture are shown in Fig. 1. The relative protein and retinol concentrations were measured by their fluorescence at 340 and 480 nm, respectively. It is evident from the disappearance of the retinol-binding protein boundary that all of the RBP migrated with the prealbumin boundary and that very little, if any, free retinol-binding protein was present. It should be noted from the distances migrated that the sedimentation rate of the complex was not much greater than that of free prealbumin.

Sucrose gradient analyses of mixtures of prealbumin and retinol-binding protein, which contained an excess of RBP, were performed in order to ascertain whether the affinity of apo- and holo-RBP for prealbumin was similar. Solutions of 47 μM retinol-binding protein were mixed with prealbumin to obtain prealbumin:RBP ratios of 0.15, 0.20, and 0.25. The sucrose gradient profiles of the protein bands are shown in Fig. 2. Although the 330:280 nm absorbance ratio varied slightly in individual tubes, the average ratio in the slower sedimenting band (corresponding to free RBP) did not change much from 0.79, the value for this retinol-binding protein preparation. If apo-RBP did not bind to prealbumin as well as holo-RBP, the absorbance ratio would have been significantly lower. Thus, this experiment indicates that there is no important difference between the affinity of apo- and holo-RBP for prealbumin.

If the binding sites of prealbumin are saturated with retinol-binding protein the faster sedimenting band which represents the complex should have absorbance ratios (330:280 nm) of 0.24, 0.37, and 0.50 for a 1:1, 2:1, and 4:1 stoichiometry, respectively. The average ratios of the fractions in the faster sedimenting boundaries (Fig. 2) represent the binding of about 2 moles of retinol-binding protein per mole of prealbumin. It is, however, evident from the incomplete resolution of the two boundaries in each gradient that redistribution of components occurred during centrifugal resolution of the free and bound retinol-binding protein. The absorbance ratios in the fractions between the major sedimenting bands at the higher prealbumin:RBP ratios have values near 0.8, which clearly indicate dissociation of the complex.

The stoichiometry of RBP binding to prealbumin was evaluated by measuring the concentration of free retinol-binding protein after separation from prealbumin and RBP-prealbumin complexes by velocity ultracentrifugation. In this method the free retinol-binding protein is uniformly distributed throughout the cell below its boundary, *i.e.* in the plateau region, and represents the equilibrium concentration of free retinol-binding protein. Fig. 3 shows the absorption profiles at 330 nm of 20 μM retinol-binding protein solutions containing increasing amounts of prealbumin, *i.e.* from 0 to 0.54 prealbumin:RBP. The free retinol-binding protein sediments at its normal rate; bound RBP sediments at a rate somewhat faster than free prealbumin, although bound and free prealbumin boundaries are not resolved. Since the sedimentation rates of retinol-binding protein and

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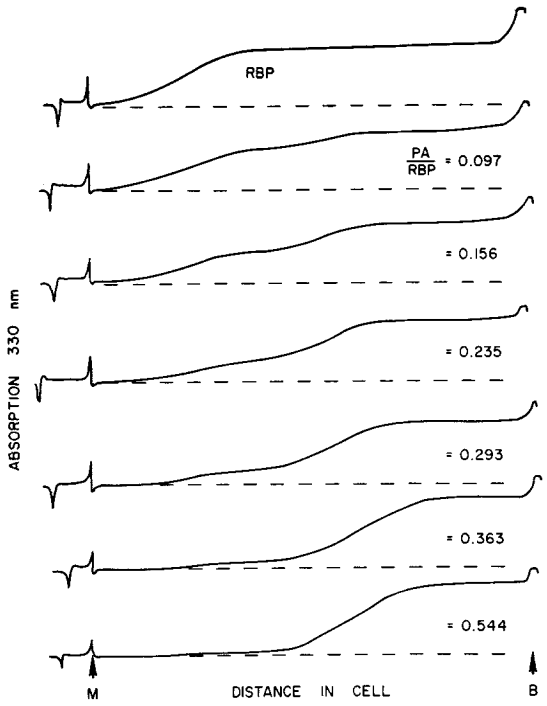

FIG. 3. Sedimentation velocity analysis of mixtures of retinol-binding protein (*RBP*) and prealbumin (*PA*) in the Spinco model E ultracentrifuge; absorbance scans were at 330 nm. *M* and *B* indicate meniscus and bottom of the solution, respectively. Protein solutions were in 0.05 M phosphate, 0.1 M KCl, pH 7.4. The mole ratio of prealbumin to retinol-binding protein in the solution before centrifugation is indicated in the figure. All solutions were 20 μM in retinol-binding protein.

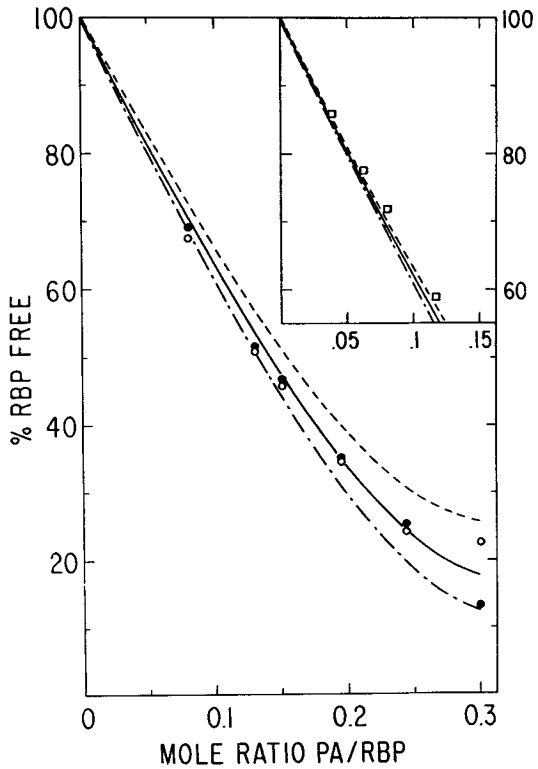

FIG. 4. Percentage of free retinol-binding protein (*RBP*) (determined from the slower moving boundary in Fig. 3) as a function of prealbumin (*PA*) concentration. Retinol-binding protein concentration, 20 μM; ● and ○, absorbance at 280 nm and 330 nm, respectively. The *inset* shows data obtained with 46 μM solutions of retinol-binding protein; absorbance was measured at 350 nm. The *lines* in both figures are theoretical binding curves for $n = 4$ and $K = 0.7 \times 10^6$ (——), 0.4×10^6 (– – –), and 1.2×10^6 (–·–·).

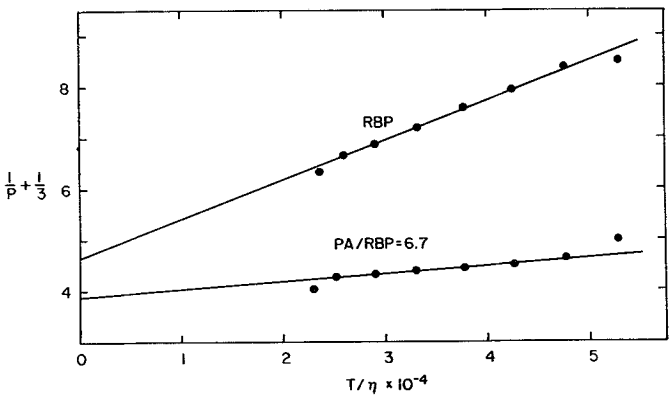

FIG. 5. Perrin plots of the polarization of fluorescence of retinol-binding protein (*RBP*). *Upper curve*, retinol-binding protein, 0.56 μM, in 0.05 M phosphate, 0.1 M KCl, pH 7.4. *Lower curve*, retinol-binding protein, 1.9 μM, and prealbumin, 12.7 μM, in the same buffer. Polarization was measured as the temperature was increased from 12 to 45°.

prealbumin are about 2.3 S and 4.6 S, respectively, the two boundaries are adequately separated (6). At the lowest prealbumin:RBP ratios, where maximal saturation of prealbumin sites occurs, there is only about a 10% increase in sedimentation rate of prealbumin. This result implies that the complex has a much greater frictional coefficient (f) than the free prealbumin. It is evident from the absorbance scans in Fig. 3 that the height of the free retinol-binding protein boundary becomes progressively smaller with increasing amounts of prealbumin and approaches zero at a prealbumin:RBP ratio near 0.5. The absorption profiles of the experiments shown in Fig. 3 were also obtained at 280 nm. The amount of free retinol-binding protein was determined from the absorbance of the slower moving boundary at 330 and 280 nm and the percentage of free RBP is shown in Fig. 4. Measurements at the two wave lengths gave similar results. The three *lines* shown in Fig. 4 represent theoretical binding curves for $n = 4$ and $K = 0.4$, 0.7, and 1.2 $\times 10^6$. Similar experiments were also performed with 46 μM retinol-binding protein solutions but were scanned at 350 nm where retinol absorption is smaller than at 330 nm. The data of four experiments (*inset* of Fig. 4) fall close to the same theoretical curves used for the smaller retinol-binding protein concentration. The values of the number of moles of RBP bound per mole of prealbumin ($\bar{\nu}$) at both retinol-binding protein concentrations are greater than 3 and consequently it is not evident whether there is any dependence of K on $\bar{\nu}$. It is difficult to measure smaller levels of prealbumin saturation by this technique since the absorption values are not readily measured with precision at the smaller concentrations needed for lesser binding.

The effect of pressure on the interaction was evaluated by centrifuging two identical solutions at 40,000 and 56,000 rpm. The concentrations of free retinol-binding protein were the same within experimental errors at the two speeds.

Polarization of Fluorescence—The polarization of the fluorescence of bound retinol can be used to provide information on the rotational diffusion behavior of retinol-binding protein. A linear dependence of $1/P$ on T/η was found for retinol-binding protein between 12 and 45° (Fig. 5). The lifetime was found to be 12 ns at 25°. The relaxation time (ρ_h), calculated from the Perrin equation and the lifetime, was 6.6×10^{-8} s at 25°. If

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retinol-binding protein were a compact sphere it would have a relaxation time (ρ_0) of 1.6×10^{-8} s. The ratio of $\rho_h:\rho_0$ is therefore close to 4.

A frictional ratio ($f:f_0$) of 1.28 has been reported for retinol-binding protein by Peterson from sedimentation and diffusion data (6). If the molecule could be represented as a prolate ellipsoid of revolution it would have an axial ratio of about 5 in the absence of hydration. If the relaxation time of retinol-binding protein was determined after random coupling with a fluorescent dye, as is customary for this type of measurement, it would be difficult to explain the high value of $\rho_h:\rho_0$ since randomly labeled monomeric native proteins normally have values between 1 and 2 (28). The rather high ratio observed for retinol-binding protein may be explained, however, if retinol is irrotationally bound to RBP and rotation about the minor axes of the molecule is mainly responsible for the observed depolarization.

The temperature dependence of the polarization of retinol fluorescence of the RBP-prealbumin complex is also shown in Fig. 5. At the high ratio of prealbumin to retinol-binding protein used (6:7:1), almost all of the RBP is bound to prealbumin, largely as the 1:1 complex. The lifetime of retinol in the complex was measured and found to be the same as that of retinol-binding protein, *i.e.* 12 ns. The calculated relaxation time of the RBP-prealbumin complex was 28×10^{-8} at 25°. The large increase in relaxation time permits one to follow the binding of retinol-binding protein to prealbumin from the change in polarization of RBP.

The effect of prealbumin on the polarization of retinol fluorescence over a 24-fold range of retinol-binding protein concentration is shown in Fig. 6. The intensity of retinol fluorescence changed only slightly (<10%) with the addition of prealbumin

The data at the three highest retinol-binding protein concentrations shown in Fig. 6 were analyzed by Equation 2 for different values of n by computing $\Sigma\Delta^2$, the sum of the squares of the differences of the best fit of the experimental points. It is clear from the values of $\Sigma\Delta^2$ shown in Fig. 7 that the data give minimum values when n is 4. The *lines* shown in Fig. 6 are theoretical binding curves using Equation 2 with n equal to 4. Table I lists the values of the binding constants and includes some data not shown in Fig. 6. The values calculated for the binding

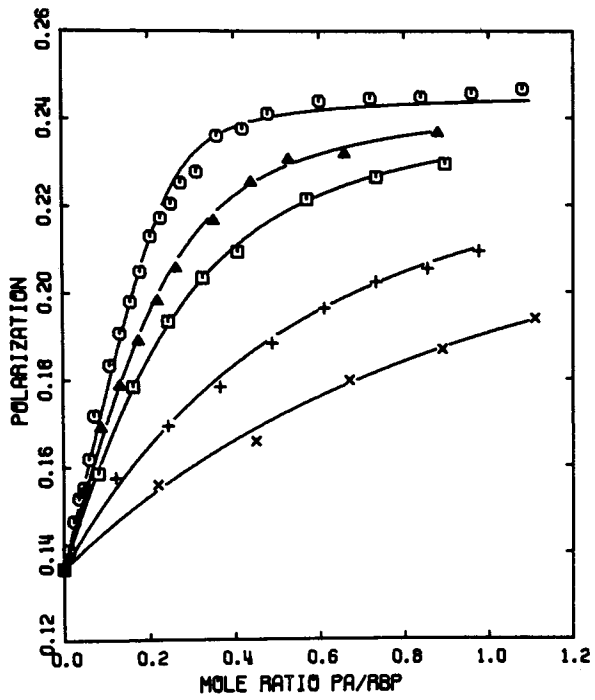

FIG. 6. Dependence of polarization of fluorescence of retinol-binding protein of (*RBP*) on prealbumin (*PA*) to retinol-binding protein molar ratio. Solvent was 0.05 M phosphate, 0.1 M KCl, pH 7.4; retinol-binding protein concentration, ○, 11.9; ▲, 3.24; □, 2.22; +, 1.01; and ×, 0.49 μM. The *lines* are theoretical best fit curves with $n = 4$ and $K = 0$, 1.69; ▲, 1.41; □, 1.49; +, 1.23; and ×, 1.11×10^6. Polarization was measured at 25°.

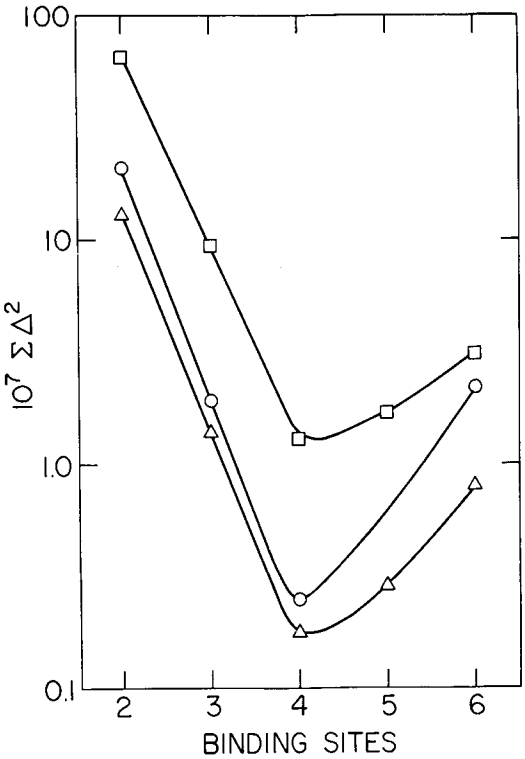

FIG. 7. Analysis of the best fit for n of data in Fig. 6. The number of retinol-binding protein binding sites (n) on prealbumin is plotted against the sum of the square of the differences ($\Sigma\Delta^2$) of the theoretical and experimental curves. Retinol-binding protein concentrations are: ○, 11.9; △, 3.24; and □, 2.22 μM.

TABLE I

Values obtained for association constant of retinol-binding protein to prealbumin at 25° from polarization of fluorescence data with $n = 4$[a]

Retinol-binding protein concentration	$K \times 10^{-6}$
μM	
11.9	1.69
5.83	1.62
3.24	1.41
2.47	1.32
2.22	1.49
1.68	1.38
1.30	1.00
1.22	1.17
1.01	1.23
0.77	1.07
0.49	1.11
0.34	1.07
Average[b]	1.23 ± 0.18

[a] See Fig. 6.

[b] The values at the two highest retinol-binding protein concentrations were not included in the calculation of the average because the small amounts of free retinol-binding protein present do not permit an accurate evaluation of K.

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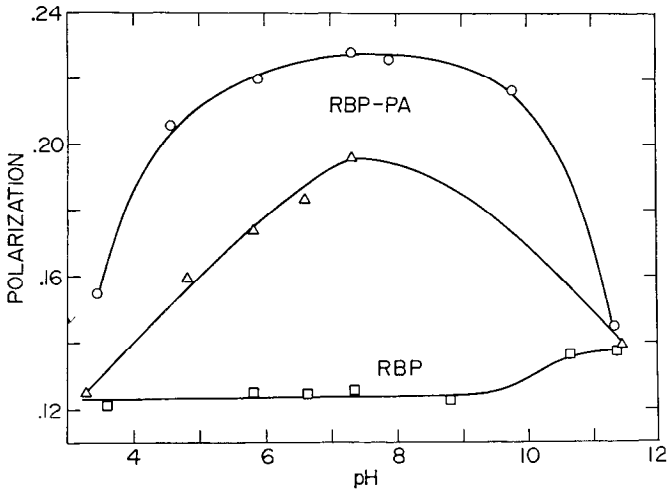

FIG. 8. The pH dependence of the polarization of fluorescence of retinol-binding protein (*RBP*). *Lower curve*, 0.64 μM retinol-binding protein in 0.05 M phosphate, 0.1 M KCl. The *upper curves* are for equimolar mixtures of retinol-binding protein and prealbumin (*PA*); ○, 6.2 μM; △, 0.62 μM, in the same buffer (T = 25°).

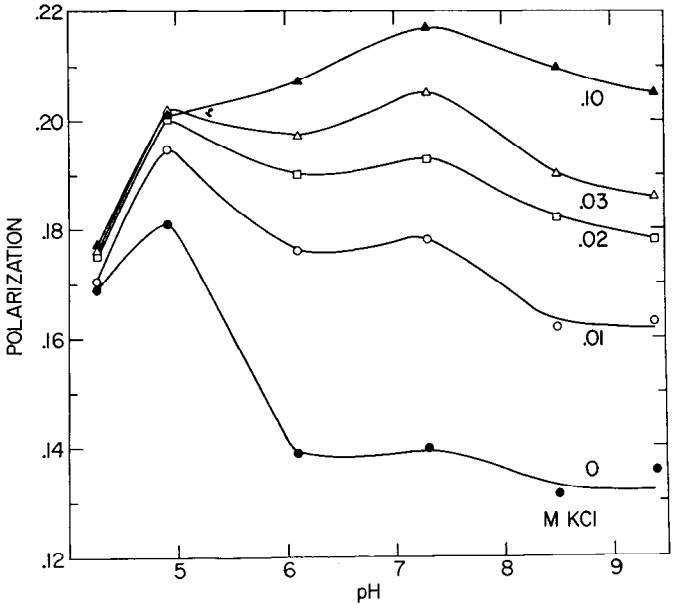

FIG. 9. The effect of KCl on the polarization of fluorescence of retinol-binding protein (3.4 μM) in the presence of prealbumin (1.7 μM) at different pH values (T = 27°). The buffers were: 2 mM acetate (pH 4.2 to 6.1), Tris (pH 7.3 to 8.5), and bicarbonate (pH 9.4).

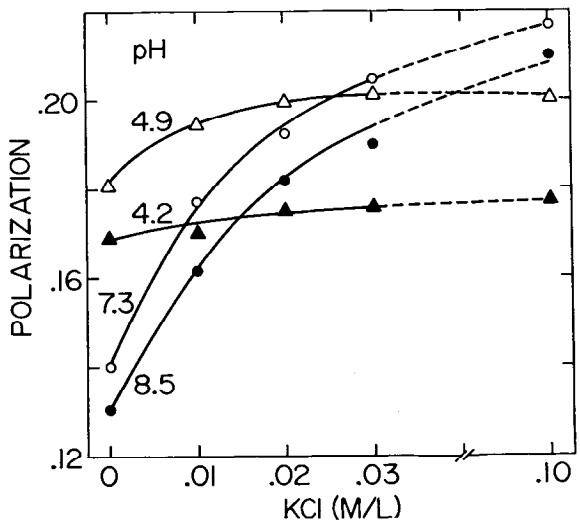

FIG. 10. The effect of KCl on the polarization of fluorescence of retinol-binding protein (3.4 μM) in the presence of prealbumin (1.7 μM) at different pH values. The buffer composition is given in the legend to Fig. 9 (T = 27°).

constant do not appear to vary significantly over a large range in retinol-binding protein concentration.

The effect of pH on the interaction between retinol-binding protein and prealbumin was also investigated by polarization. The pH dependence between 3.5 and 11.5 is shown in Fig. 8. The *upper curve* was obtained with a prealbumin:RBP ratio of 1 (RBP concentration of 6.2 μM) while the *middle curve* represents a 10-fold dilution of the same solution. The *lower curve* represents the polarization of retinol-binding protein in the absence of prealbumni. Very little change in polarization of retinol-binding protein occurs between pH 3.6 and 8.8, although a small increase occurs between pH 8.8 and 11.4. Maximum binding of retinol-binding protein is found near physiological pH, *i.e.* pH 7.4. The affinity falls regularly between pH 7.4 and 3.5 and between pH ~9 and 11.4. There is essentially no binding at 11.4 whereas at pH 3.5 a small amount remains, but only at the higher protein concentrations.

The interaction of retinol-binding protein with prealbumin is known to depend on ionic strength (6, 7). The effect of KCl on the affinity at different pH values is shown in Fig. 9. In the absence of KCl there is essentially no binding between pH 6.1 and 9.4. The polarization values increase rapidly, however, with KCl concentration to 0.1 M. At lower pH values, on the other hand, KCl has almost no influence on the interaction. At pH 4.9 there is still a small effect on KCl on polarization, but at pH 4.2 it has almost completely disappeared. The dependence of binding on KCl is shown more directly in Fig. 10, where the polarization is plotted against KCl at several pH values.

The influence of thyroxine on the affinity between retinol-binding protein and prealbumin was evaluated since thyroxine is bound very strongly to prealbumin (2, 24, 25). Two solutions of identical amounts of prealbumin, one containing 1 mole of thyroxine per mole of prealbumin (98% bound), and the second without thyroxine, were added to identical solutions of retinol-binding protein. The fraction of thyroxine bound to prealbumin varied from 85% at the lowest to 94% at the highest prealbumin concentration.[2] Thyroxine had essentially no effect on the binding of retinol-binding protein by prealbumin, as seen in Fig. 11. At the highest prealbumin:RBP ratio, the polarization values of the two solutions differed only slightly. Furthermore, the dependence of the interaction on pH between pH 7.4 and 3.4 was also unaffected by thyroxine (Fig. 11). Thyroxine is strongly bound to prealbumin at least to pH values as low as 3.5.[2]

[2] A value of 1.2×10^8 was used for the equilibrium constant of binding of thyroxine to prealbumin (R. A. Pages, J. Robbins, and H. Edelhoch, manuscript in preparation).

DISCUSSION

The retinol-binding protein preparation used in this study had a 330:280 nm absorbance ratio of 0.79. According to previous data (1), about 75% of the protein of this preparation should contain retinol (holo-RBP), the rest being devoid of retinol (apo-RBP). Since we have used the absorbance and fluores-

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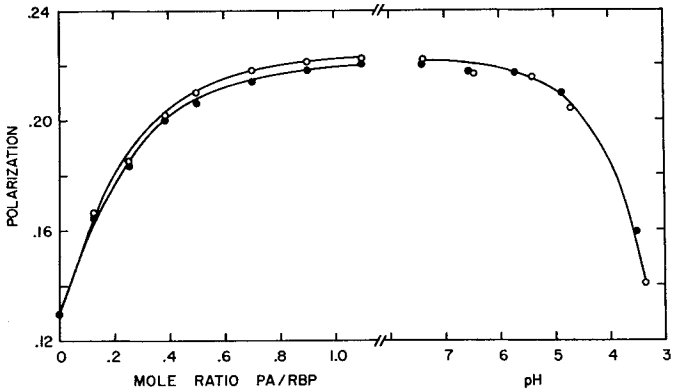

FIG. 11. The effect of thyroxine on the binding of retinol-binding protein (*RBP*) to prealbumin (*PA*) and on the pH dependence of the polarization of the complex. Retinol-binding protein solutions (2.8 μM) in 0.05 M phosphate + 0.1 M KCl, pH 7.4, were titrated with prealbumin (○) and prealbumin (●) containing 1 mole of thyroxine per mole of prealbumin. At the final prealbumin to retinol-binding protein mole ratio the solutions were acidified with HCl (T = 28°).

cence of retinol for measuring its interaction with prealbumin, we have ascertained that the binding of retinol-binding protein to prealbumin does not depend on the presence of retinol. The sucrose density gradient experiments (Figs. 1 and 2) showed that both forms of retinol-binding protein bind to prealbumin. The binding of apo-RBP to prealbumin has been demonstrated previously by gel filtration (3) and fluorescence quenching experiments (10).

The binding of retinol-binding protein by prealbumin was investigated by velocity centrifugation which takes advantage of the greater sedimentation rates of prealbumin and RBP-prealbumin complexes compared to free retinol-binding proteins. With increasing binding of RBP the sedimentation rates of the complexes increase slightly. Sedimentation values of 2.1 S (3) and of 2.3 S (6) have been reported for retinol-binding protein, and of 4.6 S (3) and 5.6 S (6) for the RBP-prealbumin complex. The amount of free retinol-binding protein was obtained from the height of either the 330 nm or the 280 nm absorbance curves of the slower sedimenting boundary. This concentration of free retinol-binding protein is uniformly distributed throughout the cell and therefore represents the equilibrium value of free RBP. These experiments were all performed at very low protein concentrations (~0.5 mg per ml) where the activities of the various protein species should be equal to their concentrations and corrections for Johnston-Ogston effects are negligible. The data shown in Fig. 4 were obtained with 20 μM retinol-binding protein solutions and the points fall on a binding curve for four equivalent sites with $K = 0.7 \times 10^6$. The *dotted lines* are curves calculated for $n = 4$ and association constants of 0.4×10^6 and 1.2×10^6. It is clear from the small differences between the theoretical lines that K is not very sensitive to the concentration of free retinol-binding protein. The number of moles of retinol-binding protein bound per mole of prealbumin was in all cases greater than 3 and reached a maximum value of 3.9.

The polarization technique was employed in order to measure the binding at concentrations of retinol-binding protein which cover the entire range of site saturation. This method is particularly applicable in this study since retinol is a natural component of retinol-binding protein and synthetic conjugation of a fluorescent dye is not necessary. The rather large change in polarization when RBP binds to prealbumin permits considerable precision in determining the fraction of retinol-binding protein which is bound. Since the value of the polarization of the 1:1 complex is close to P_0, the values of higher complexes cannot be much different. The large increase in polarization results from a large change in relaxation time. The ratio of relaxation times, *i.e.* $\rho_h:\rho_0$, of the 1:1 complex is about 4 and is unusually large. The sedimentation rate of prealbumin does not increase very much with complex formation, indicating that the frictional coefficient of the complex increases appreciably with size. This result leads to increasing values of the frictional ratio, $f:f_0$, where f_0 is the frictional coefficient of the complex if it were spherical. The high ratios of $\rho_h:\rho_0$ and $f:f_0$ can be explained if the RBP-prealbumin complex is much less symmetrical than either component. Moreover, the asymmetry of the higher complexes should increase with increasing size since the sedimentation rate changes only trivially. The apparent increase in asymmetry with binding presumably arises from increasing occlusion of water molecules in the area near the interfaces with increasing size of the complex.

We have not found as large an increase in retinol fluorescence as reported by Peterson and Rask (10). It was more recently found that the intensity of retinol fluorescence increased only about 15% on binding of retinol-binding protein to prealbumin (29). The polarization values have been used directly to evaluate the binding constant since retinol fluorescence intensities of the bound and free RBP differ only slightly and they can be equated to the anisotropy values used by Rawitch and Weber (30), $A = 2/3\ (1/P + 1/3)^{-1}$, since $1/P$ is much greater than 1/3. The results obtained in the polarization of fluorescence studies (Figs. 6 and 7) suggest that there are four binding sites of prealbumin for retinol-binding protein, each with an apparent association constant of approximately 1.2×10^6. This value is similar to that obtained by the totally different technique of velocity ultracentrifugation.

Since both procedures give nearly identical association constants, it seems that prealbumin contains four binding sites for retinol-binding protein, at least in the preparations studied. The question may then be raised as to why previous investigations of the interaction of retinol-binding protein with prealbumin have not recognized the existence of more than one RBP-binding site on prealbumin. Part of the explanation probably derives from the fact that in plasma, retinol-binding protein normally circulates largely as a 1:1 molar complex with prealbumin, with the molar concentration of retinol-binding protein slightly less than one-half that of prealbumin (31). In addition, previous studies of this interaction have generally employed very dilute solutions, in which case some dissociation of the 1:1 complex, and very little formation of higher complexes, might be expected from the presently estimated values for n and K.

The interaction of retinol-binding protein with prealbumin is strongly pH-dependent (Fig. 8). The maximum binding occurs near physiological pH and falls gradually at lower and higher pH values. Prealbumin is stable in concentrated urea and guanidine solutions and at pH values as high as 12 (16, 19). It does, however, undergo a structural transition below pH 3.5. Retinol-binding protein appears to be stable between pH 3.6 and ~9 judging from the polarization values. The weaker association that occurs between pH 6 and 3.5 cannot, therefore, be attributed to structural changes in either protein, although minor alterations may have escaped detection by the methods employed. There appears to be little difference in affinity between pH 7.4 and 8.5, but by pH 10.5 the interaction is much weaker. These results confirm and extend previous gel filtration studies (3) which showed that the RBP-prealbumin complex was

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stable in the pH range 5.8 to 7.5, but that considerable dissociation of the complex occurred as the pH was raised from 8.6 to 10.3. The RBP-prealbumin complex is also completely disrupted in 6 M urea (3). The large decrease at pH 10.5 may be due to modifications in the conformation of retinol-binding protein since there is an increase in its polarization at pH 10.5 (Fig. 10), but more data are needed to establish this point.

The interaction of retinol-binding protein with prealbumin is very sensitive to ionic strength; in fact, this feature has been used to separate RBP from prealbumin (6, 7). The results shown in Fig. 9 clearly demonstrate the sensitivity of binding to KCl concentration at neutral and higher pH values. However, at pH 4.2 the binding of RBP to prealbumin is virtually independent of salt. Peterson and Berggard have reported an isoelectric point of retinol-binding protein near 4.6 (5). The value for prealbumin is probably near pH 4.[3] At pH 4.2, therefore, both proteins are near their isoelectric state and binding is independent of KCl concentration. Electrostatic interactions between the two negatively charged proteins at higher pH values may be responsible for the effect of salt on the binding constant.

Thyroxine is strongly bound to prealbumin (2, 32, 33). It was therefore of interest to see whether the binding of the second ligand of prealbumin, *i.e.* retinol-binding protein, would be affected by thyroxine. The results of this and other studies (2) indicate no interdependence between retinol-binding protein and thyroxine binding to prealbumin.

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[3] R. Ferguson, unpublished results.

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