Combination of Specific Antibodies with the Human Vitamin A-transporting Protein Complex*

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SUMMARY

The interactions of specific antibodies with the human retinol-binding protein (RBP) and the thyroxine-binding prealbumin have been investigated. When an antibody combines with either of these proteins, the tryptophyl fluorescence of the antibody is partially quenched. Polarization of fluorescence of RBP-retinol increased rapidly on formation of antibody-RBP complexes. With fluorescence-quenching and polarization measurements as indicators, the stoichiometries and equilibrium constants of the antigen-antibody reactions have been studied.

The data show that RBP, whether free or in complex with prealbumin, exhibits identical reactivity with anti-RBP Fab'fragments. The conclusion is reached that the prealbumin binding site of RBP is not a major antigenic structure. The data indicate that the number of antigenic sites on RBP is limited and is the factor which controls the number of antibodies bound per RBP molecule. By quantum yield measurements of retinol it was found that RBP does not undergo any measureable conformational change on complex formation with Fab'fragments.

Free prealbumin could simultaneously interact with a maximum number of 12 anti-prealbumin Fab'fragments. This suggests that there are three antigenic sites on each of the four identical prealbumin subunits. RBP, on forming a complex with prealbumin, competed with four anti-prealbumin Fab'fragments. These Fab'fragments showed a limited heterogeneity indicating that they may be directed toward a single antigenic site. It is proposed that the competition between RBP and the anti-prealbumin Fab'fragments may be interpreted as a consequence of a negative cooperativity on RBP forming a complex with prealbumin.

The equilibrium constant for Fab'fragments forming a complex with prealbumin was lowered on thyroxine binding to prealbumin. This result supports the earlier suggestion that thyroxine binding to prealbumin is an example of a negative homotropic interaction.

Antibodies are known to react in a highly specific manner with their antigen. According to current concepts the antibody combining sites are complementary to the patterns of the antigenic determinants and conformational changes of an antigenic site should thus give rise to observable perturbations in the antigen-antibody reaction. On the other hand, accumulating evidence (1-5) suggests that antibodies may also induce conformational changes in the antigen. The use of immunological techniques thus provides a powerful tool to establish conformational identity and to detect changes in conformation.

We report here the application of immunological techniques to the study of complexes of antibodies and the human vitamin A-transporting protein complex (6-8). Since the constituents of the protein complex, the retinol-binding protein and prealbumin, are able to bind ligands, retinol and thyroxine, respectively, which quench protein tryptophyl fluorescence, we employed fluorescence measurements to determine the effects of the interaction of specific monoclonal antibodies with the antigens. Furthermore, retinol is a natural fluorescent reporter group bound to a single specific site in RBP, the fluorescence of which may give information about the structure of its environment in the protein. The data obtained in these studies indicate that the structure of RBP is not altered on complex formation either with prealbumin or with specific Fab'fragments. The interaction between prealbumin and thyroxine, on the other hand, perturbed the prealbumin complex forming with specific monovalent antibodies.

EXPERIMENTAL PROCEDURE

Materials

Proteins Prealbumin was isolated as described elsewhere (10). The prealbumin-RBP complex was isolated according to a published procedure (8). RBP was isolated from the prealbumin-RBP complex (10) or from urine of patients with tubular proteinuria by means of affinity chromatography on a prealbumin-coupled Sepharose column (11).

Other Materials—Sephadex G-100 and G-200 and Sepharose 4B, products of Pharmacia Fine Chemicals, were used according to the instructions supplied by the manufacturer. All other chemicals were the best available grade from commercial sources.

Methods

Preparation of Fab'fragments—The antisera were prepared in rabbits by a schedule of injections previously described (12). Specific antibodies were prepared in the following way. The

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antisera were mixed with agarose immunoabsorbents. These immunoabsorbents were prepared by binding of prealbumin or RBP to Sepharose 4B (13) according to the method of Cuatrecasas (14). In a typical preparation, antigen-Sepharose immunoabsorbents were prepared by binding of prealbumin or RBP to Sepharose 4B (13) according to the method of Cuatrecasas (14). The adsorbed antibodies were eluted with 0.2 M glycine HCl buffer, pH 2.9, and the eluate was immediately titrated to pH 8.0 with 1.0 M Tris. By this procedure between 1 to 2 mg of specific antibodies were obtained per ml of antisera.

To avoid the complication in the fluorescence experiments of turbidity resulting from precipitate formation, the purified antibodies were digested with 1% by weight of pepsin at pH 4.5 as described by Nisonoff et al. (15). The digestion was stopped by raising the pH to 8 with 1 M Tris. The digest was then applied on a Sephadex G-200 column (2 x 130 cm), equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, to isolate (Fab')2-fragments free from undigested immunoglobulin. Monovalent Fab'-fragments were prepared from the (Fab')2-fragments by reduction with 0.01 M dithiothreitol for 1 hour and alkylation with 0.022 M iodoacetamide in the dark for 30 min. The Fab'-fragments were freed from excess reagents and small amounts of aggregated protein by means of gel chromatography on Sephadex G 100 equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Part of the highly purified Fab'-fragments was then applied to an immunoabsorbent column to test their antigen-binding activity. The adsorbed fragments were eluted with 0.2 M glycine HCl buffer, pH 2.9. Approximately 95% of both the anti-RBP and the anti-prealbumin Fab'-fragments were capable of binding specific antigen as judged by this method.

Fluorescence Measurements—Most measurements were carried out with an Aminco-Bowman spectrophotofluorometer. Polarized fluorescence was estimated in a Zeiss ZFMAC spectrophotofluorometer equipped with double monochromators. Fluorescence was maximal when the excitation wavelength was 285 nm and the fluorescence at 340 nm was measured. Therefore, this combination of wave lengths was used for protein fluorescence measurements. No correction was made for the wave length dependence of either the light source or the photomultiplier output. Protein concentrations were low enough so that there was little absorption of either the exciting or fluorescence radiation. All measurements were carried out at room temperature (23 ± 2°). Quantum yields of retinol fluorescence were estimated in a Zeiss ZFM4C spectrophotofluorometer. On rearrangement of the mass law derived from Sips' equation (18, 19) (cf. Equation 1)

\[
F_{b \text{max}} = \frac{1}{\beta x (1 - \beta) x} + 1
\]

where \(F_{b \text{max}}\) is the maximum value of the molar concentration of the bound fluorescent ligand, and \(x\) is the total concentration of the fluorescent ligand.

When prealbumin, Fab'-fragments against prealbumin, and RBP are simultaneously present, there will be a competition between Fab' and RBP for the sites of prealbumin. The mass law for the competition-type experiment is hence

\[
\frac{F_{b \text{max}}}{\beta x} = \frac{1/(K_{o}(1 - \beta) x)}{K_{r} x(1 - \beta) x} + 1
\]

where \(d\) is the effective concentration of Fab'-fragments, i.e. the concentration divided by the stoichiometric reaction number, and \(K_{r}\) the apparent association constant of prealbumin and RBP.

Ultracentrifugations—Molecular weights were determined at 20° in a Spinco model E analytical ultracentrifuge equipped with an RRTC (temperature control unit) and an electronic speed control. All samples were dissolved in the appropriate buffers and dialyzed in the cold against two changes of the solvent. Densities were determined by pycnometer. Six-channel Epon-filled Yphantis centerpieces and sapphire windows were used throughout. Recordings were made with the photoelectric scanning system set at 280 or 330 nm. The sedimentation equilibrium experiments were performed by means of the low speed method of Richards and Schachman (22). Speed settings and equilibrium times were estimated as described by Teller et al. (23). The ex
periments were discontinued when no redistribution of material could be observed over a period of several hours.

Calculations of apparent weight average molecular weights were computed from the following equation (22).

$$M_w = \frac{2kT(d \ln C/dx^2)}{(1-\bar{v}p)\rho}$$

where the symbols have their usual meaning. The value 0.72 was used for the partial specific volume of RBP and Fab'-fragments (7). Local weight average molecular weights were obtained as described by Yphantis (24) over five equally spaced $x$ coordinates.

**Immunochemical Methods**—Ouchterlony immunodiffusion analyses (25) were carried out as described elsewhere (8). Quantitative precipitin analyses of prealbumin, RBP, and the prealbumin-RBP complex were performed with anti-prealbumin and anti-RBP sera, respectively. Portions (200 µl) of these antisera, diluted 1:5, were incubated with various amounts of the antigens. The mixtures were allowed to react for 2 h at 25° followed by measuring the absorbance at 280 nm. The precipitates were collected by centrifugation at 15,000 x g for 30 min. The supernatants were discarded whereas the precipitates were washed repeatedly with ice-cold 0.15 µ NaCl. The washed and dried precipitates were dissolved in 0.5 µ NaOH and the protein content was estimated by measuring the absorbance at 280 nm.

**Other Methods**—Affinity chromatography of RBP on prealbumin-coupled Sepharose was accomplished as described elsewhere (11). Estimates of the affinity between prealbumin and thyroxine were performed by use of the Colowick and Womack method of rate of dialysis (26). The details have been given elsewhere (27).

Protein and retinol concentrations were determined by relating the absorbance at 280 and 330 nm, respectively, to the relevant extinction coefficients (8). Diluted protein solutions were concentrated by ultrafiltration (28) with use of the Visking dialysis tubing, 23 x 32 inches (Union Carbide Corp., Chicago, Ill.), as the ultrafiltration membrane (29). The negative pressure was not allowed to exceed 400 mm Hg in order to obtain protein recoveries of 85% or more.

**RESULTS**

**Antigenic Reactivity of Prealbumin, RBP, and Prealbumin-RBP Protein Complex**—With use of specific antisera directed against RBP and prealbumin, respectively, the immunological reactivity of the individual proteins and of the prealbumin-RBP complex was investigated. The quantitative precipitin technique was employed and the results are summarized in Fig. 1. It is evident from the figure that RBP when free exhibited the same antigenic characteristics as when bound to prealbumin. However, a clear difference was noted between free and complex-bound prealbumin. The precipitin curves suggest that the prealbumin-RBP complex reacts with a smaller number of antibodies than free prealbumin.

The results from the quantitative precipitin experiments were corroborated by binding of the antibodies to insolubilized antigen. Table I shows that prealbumin could bind a greater number of monovalent prealbumin antibodies than could prealbumin-RBP. Virtually identical binding behavior was noted when monovalent RBP antibodies were reacted with either immobilized RBP or prealbumin-RBP (Table I). It is, however, difficult to investigate the reaction stoichiometry from these experiments, since it has been shown that only a fraction of the immobilized antigen is reactive (11).

![Fig. 1. Quantitative precipitin curves of prealbumin (□) and the prealbumin-RBP complex (●) with use of an anti-prealbumin serum and of RBP (□) and the prealbumin-RBP complex (●) with use of an anti-RBP serum. The details are outlined under "Methods."](http://www.jbc.org/)

**TABLE I**

| Immobilized antigen | Antibody specificity | Molar ratio of bound antibody to antigen |
|---------------------|---------------------|----------------------------------------|
| Prealbumin          | Prealbumin          | 4.2                                    |
| Prealbumin "complex"* | Prealbumin         | 2.8                                    |
| RBP                 | RBP                 | 1.1                                    |
| RBP "complex"*      | RBP                 | 1.2                                    |

* "Complex" denotes that prior to addition of Fab'-fragments, RBP (or prealbumin) was added to the prealbumin (or RBP)-coupled Sepharose to form a noncovalent interaction with the matrix-bound protein. The amount of protein added was sufficient to saturate all accessible binding sites on the protein coupled to Sepharose.

It is obvious from the above results that RBP and anti-prealbumin Fab'-fragments compete for similar binding sites on prealbumin. To isolate the specifically competing Fab'-fraction a column of prealbumin-coupled Sepharose was saturated with anti-prealbumin Fab'-fragments. After having washed the column with the equilibrating buffer until the eluate was virtually devoid of protein, RBP was applied. It can be seen from Fig. 2 that Fab'-fragments were displaced from the column by RBP. On lowering the ionic strength of the eluting buffer the prealbumin-bound RBP was eluted from the column, and by lowering the pH, the remaining Fab'-fragments were released (Fig. 2). The two Fab'-fragments fractions, henceforth called Fab' I and Fab' II, were subsequently used for the fluorescence measurements (see below). Fab' I fragments were separated from RBP by gel chromatography on Sephadex G-100 in 0.02 µ Tris-HCl buffer, pH 7.4, containing 0.15 µ NaCl, and concentrated by ultrafiltration.

**Effects of Antibodies on Retinol and Thyroxine Binding**—The quantum yield of the retinol fluorescence of free RBP increases by 50% when RBP is bound to prealbumin (17). To investigate whether Fab' fragments directed against RBP exerted a similar
The values obtained from the quantum yield measurements were
identical (0.04) within experimental error irrespective of the
presence or absence of Fab'-fragments.

It is well known that the fluorescence of retinol decays with
time, probably due to molecular alterations under the influence of
ultraviolet light. Fig. 3 shows the effect of prolonged irradiation
on the retinol fluorescence. It is evident from the figure that
prealbumin, when bound to RBP, shields the vitamin moiety in
the microenvironment of retinol.

Effect the quantum yield of the retinol fluorescence was measured
for mixtures of Fab'-fragments and RBP in varying molar ratios.
The values obtained from the quantum yield measurements were
identical (0.04) within experimental error irrespective of the
presence or absence of Fab'-fragments.

It is well known that the fluorescence of retinol decays with
time, probably due to molecular alterations under the influence of
ultraviolet light. Fig. 3 shows the effect of prolonged irradiation
on the retinol fluorescence. It is evident from the figure that
prealbumin, when bound to RBP, shields the vitamin moiety in
part from the radiative effects. Fab'-fragments directed against RBP, on the other hand, do not seem to exert any effect of
similar kind (Fig. 3). Within the limit of the techniques employed,
it may thus be concluded that Fab'-fragments bound to
RBP neither affect the prealbumin-RBP interaction nor influence
the microenvironment of retinol.

Since prealbumin binds thyroxin, its affinity for thyroxin was investigated in the presence of specific Fab'-fragments. By rate of dialysis it was shown that the apparent association
constant of thyroxine and the high affinity site of prealbumin
(27) appeared identical in the absence of Fab'-fragments (1.9 ×
10^7 M^-1) and the presence of saturating amounts of the monova-
 lent antibodies (1.7 × 10^7 M^-1). This result suggests that the
Fab'-fragments do not induce any conformational change of pre-
albumin affecting its interaction with thyroxine.

**Stoichiometric Estimations**—Aliquots of a solution of anti-RBP
Fab'-fragments were titrated with RBP or with the prealbumin-
RBP complex. The results are shown in Fig. 4. The percentage
of the maximum of the quenched fluorescence is plotted as a func-
tion of the ratio of the antigen concentration to the total antibody
concentration. The line of the final fluorescence quenching at
complete reaction intersects the initial slope of the plot at an anti-
gen to antibody ratio of approximately 0.25 both for RBP and
prealbumin-RBP. This implies an average total binding of 4
Fab' molecules per RBP molecule. Due to the specific absorb-
cence of retinol at 330 nm it was possible to measure the reaction
stoichiometry of Fab'-fragments and RBP also by sedimentation
ultracentrifugation. In separate experiments it was
found that both RBP and Fab'-fragments appeared homogeneous
with weight average molecular weights of 21,000 and 46,000, re-
spectively. Mixtures of Fab'-fragments and RBP (within the
molar range of 8 to 1 for the initial concentrations of the indi-
vidual components) showed, as expected, a heterogeneous be-

davior in the ultracentrifuge. At high ratios of Fab'-fragments
to RBP, the limiting local weight average molecular weight ap-
proached a value of 200,000, close to the expected theoretical
value of 205,000 for a reaction stoichiometry of 4:1. Both
fluorescence measurements and sedimentation ultracentrifuga-
tion thus gave similar results.

The titrations of anti-prealbumin Fab'-fragments with thyroxin-containing prealbumin gave plots of qualitatively similar appearance as that shown for RBP (cf. Fig. 4). The calculated
reaction stoichiometry is presented in Table II. As can be seen in
the table an average total binding of 3 Fab' molecules per
prealbumin subunit (12 per prealbumin molecule) was obtained.
Fab' I and Fab' II fragments exhibited an average maximal bind-
ing of 4 and 8 molecules, respectively, per prealbumin molecule.
These data then suggest that RBP compete with Fab'-fragments
directed against one-third of the prealbumin antigenic deter-
minants.

**Equilibrium Constants for Fab'-fragments and RBP, Prealbumin,**
and Prealbumin-RBP—The equilibrium constants for the various reactions were calculated from the fluorescence-quenching experiments described above, or, for reactions involving RBP, by polarization of the retinol fluorescence. The polarization of the fluorescence of RBP-retinol increased rapidly as specific Fab'-fragments were added to the system (Fig. 5) and the polarization approached a limiting value of 0.385. The corresponding value for the prealbumin-RBP retinol complex, when saturated with Fab'-fragments, was 0.40. The fluorescence spectra of RBP-retinol and prealbumin-RBP-retinol and of their corresponding Fab'-fragments were added to the system (Fig. 5) and the polarization of the retinol fluorescence. The polarization of the fluorescence of RBP-retinol increased rapidly as specific Fab'-fragments were added to the system (Fig. 5) and the polarization increased as a function of added anti-RBP Fab'-fragments.

TABLE II

| Monovalent antibody | Antigen                  | \( K_0 \) | \( a \) | \( x \)  | Method |
|---------------------|--------------------------|----------|--------|--------|--------|
| Anti-RBP            | RBP                      | 3.0      | 0.63   | 4.3    | P      |
| Anti-RBP            | Prealbumin-RBP           | 3.0      | 0.63   | 4.3    | P      |
| Anti-prealbumin     | Prealbumin-RBP           | 2.7      | 0.65   | 4.0    | Q      |
| Anti-prealbumin     | Prealbumin-thyroxine     | 3.3      | 0.78   | 4.2    | Q      |
| Fab' I              | Prealbumin-thyroxine     | 3.0      | 0.80   | 5.3    | P      |
| Fab' II             | Prealbumin-thyroxine     | 0.63     | 0.68   | 8.1    | Q      |
| Anti-prealbumin     | Prealbumin-RBP           | 3.0      | 0.65   | 8.3    | P      |
| Fab' II             | RBP                      | 2.2      | 0.61   | 7.9    | Q      |

- A Heterogeneity index.
- *Moles of Fab'-fragments bound per mole of antigen.
- 'Fluorescence polarization and fluorescence quenching are denoted by P and Q, respectively.

**Fig. 5.** The polarization of fluorescence of RBP-retinol (1.0 \( \times 10^{-6} \) M) as a function of added anti-RBP Fab'-fragments (O). The experiment was carried out at room temperature in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Excitation was at 330 nm and emission was measured at 470 nm. Anti-prealbumin Fab'-fragments (●) substituted anti-RBP Fab'-fragments in an identical experiment.

**Fig. 6.** The fraction of RBP-retinol bound (\( \beta \)) as a function of added anti-RBP Fab'-fragments. Experimental conditions were as given for Fig. 5. RBP-retinol (○); prealbumin-RBP-retinol, 1.0 \( \times 10^{-6} \) M (●).

**Fig. 7.** Sips' plot of fluorescence-quenching data for the prealbumin anti-prealbumin system according to Equation 1. Anti-prealbumin Fab' II fragments (2 \( \times 10^{-7} \) M) were titrated with increasing amounts of prealbumin-thyroxine (●) and prealbumin-RBP complex (○). The straight lines shown represent the best least squares fit of the data and correspond to \( K_0 = 6.3 \times 10^{4} \) M\(^{-1}\) and \( a = 0.68 \) for prealbumin-thyroxine and to \( K_0 = 3.0 \times 10^{-7} \) M\(^{-1}\) and \( a = 0.65 \) for prealbumin-RBP.
The competition curve was calculated from Equation 7. The best square values of the parameters determined from the experimental data were used to construct the curves shown. Values of the parameters were: \( K_0 = 3.0 \times 10^9 \text{ M}^{-1}; \ a = 0.80; \ K_0 \max = 5.0 \times 10^{-7} \text{ M}; \ R = 1.5; \ d = 1.0 \times 10^{-7} \text{ M}; \ K_R = 2.0 \times 10^8 \text{ M}^{-1} \).

They found excellent agreement with the corresponding values obtained by fluorescence quenching (cf. Table II).

**Discussion**

The human vitamin A-transporting protein complex is intriguing in view of the many molecular interactions pertaining to this system. Since antibodies against prealbumin and RBP are not cross-reacting and are directed against various sites on the two proteins, they seemed to be suitable tools for evaluation of some of the characteristics of the binding processes. An inherent limitation to this approach is, however, that the antibodies, formed in rabbits, are produced only against antigenic structures of the injected proteins differing from the endogenous counterparts of the animal. This means that there are two possible factors controlling the stoichiometry of the binding of antibodies to the antigen (32). The first is a limitation imposed by there being a finite number of antigenic sites. The second is a steric restriction; if enough antibodies bind to the antigen, they will completely cover its surface, making it impossible for other antibodies to approach. In the latter case the number of antibody binding sites would exceed the binding stoichiometry. The former alternative gains support from the findings that antibodies against RBP react identically with the free antigen and the prealbumin-RBP complex. Prealbumin, which is somewhat larger than the Fab'-fragments, binds to RBP irrespective of the presence of anti-RBP Fab'-fragments and it may thus be concluded that the prealbumin binding site of RBP is not antigenic.

The lack of antigenicity of the prealbumin binding site of RBP may be explained assuming that the prealbumin binding site of RBP has been conserved during evolution (33). Amino acid substitutions in this region of RBP may require complementary mutations in the gene for prealbumin to yield proteins with sustained ability to interact.

The great specificity in the interaction of RBP and prealbumin is strikingly shown by the fluorescence experiments reported here. The average of four simultaneously bound Fab'-fragments to RBP do not appreciably change the fluorescence characteristics of retinol compared to free RBP-retinol whereas the interaction of prealbumin and RBP causes profound effects. The equilibrium constants for RBP and each of the two types of proteins are of similar magnitude indicating that the differences encountered for the binding of prealbumin and Fab'-fragments are, respectively, not merely related to binding strength. Prealbumin could simultaneously bind an average of 12 Fab'-fragments. Since prealbumin is composed of four identical subunits (10) it may be inferred that there are three independent antigenic sites per polypeptide subunit. These numbers are identical with those found for hemoglobin (32) which has about the same molecular size as prealbumin. In view of the molecular symmetry of the arrangement of the prealbumin subunits (34) it is conspicuous that RBP compete with four of the Fab'-fragments for binding to prealbumin. A possible interpretation for this observation is that the RBP binding site of prealbumin corresponds to one or two of the antigenic sites and that RBP binds to prealbumin in a negatively cooperative manner. Accordingly, this means that the prealbumin molecule contains multiple (two or four) RBP binding sites. On forming a complex with RBP, a conformational change in prealbumin could alter the corresponding antigenic sites so that they no longer are recognized by the antibodies. Recent results obtained in this laboratory give support to this view since it has been found that the free prealbumin subunit can interact with RBP. Furthermore, the index of heterogeneity (a) is significantly higher for the monovalent antibodies competing with RBP than for the rest of the anti-prealbumin Fab'-fragments. This limited heterogeneity may point to the fact, that the anti-prealbumin Fab'-I fragment are directed against a single antigenic determinant.

The anti-prealbumin Fab'-II fragments reacted with eight sites on prealbumin. It is interesting to note that the equilibrium constant for the reaction was dependent on the presence of thyroxine. We had suggested earlier (27) and shown recently that thyroxine binds to prealbumin with a negative cooperativity. This invokes that after 1 molecule of thyroxine has formed a complex with a prealbumin subunit, the thyroxine binding sites of the other subunit should change their structure so that further complex formation of thyroxine with prealbumin should be impeded. The lowered affinity of the Fab'-II fragments for prealbumin may thus be interpreted as a result of a slight conformational change in part of the prealbumin molecule. It may be inferred that the suggested conformational change is not a result of thyroxine binding but RBP binding to prealbumin. This was, however, excluded by performing the titration in the presence of both thyroxine and RBP. The affinity constant obtained was similar to that found when prealbumin-thyroxine was used as the antigen. There is no direct competition between thyroxine and the Fab'-II fragments since the high affinity thyroxine binding site of prealbumin exhibits the same equilibrium constant for thyroxine whether a large excess of anti-prealbumin Fab'-fragments are present or not. Assuming that a conformational change occurs in prealbumin on forming a complex with thyroxine, this means that only part of the molecule is altered. This can be deduced from the fact that RBP complexes with prealbumin irrespective of its thyroxine binding status (27, 35). Furthermore, the binding characteristics for the Fab'-I fragments are obviously not perturbed by the presence of thyroxine bound to prealbumin.

The generality of the differences encountered has not been established. The antibodies were obtained from pools of three animals. The different reactivity of Fab'-II fragments toward prealbumin with and without thyroxine may not be a general phenomenon. Similarly, other details of our results such as equilibrium constants and the magnitudes of the differences found for the binding of a given antibody preparation to different antigens may well vary from one preparation to the next. However, the ability of fluorescence methods to detect small changes in...
antibody binding and differences in equilibrium constants that result from small conformational changes in the structure of an antigen has been amply shown.

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