Comparative Studies on the Vitamin A Transporting Protein Complex in Human and Cynomolgus Plasma†

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ABSTRACT: The vitamin A transporting protein complex has been isolated from serum of cynomolgus monkeys. The complex components, retinol-binding protein (RBP) and thyroxine-binding prealbumin, have been studied separately and together by physical–chemical, chemical, and immunological techniques and the results have been compared to those of the human counterparts. By several physical–chemical and chemical criteria RBP and prealbumin of the two species appeared indistinguishable. On immunological analyses and on electrophoresis RBP from both species exhibited complete identity, whereas differences were evident for prealbumin. The only differences encountered for RBP of the two species seem to be two amino acid substitutions, both of which can be brought about by single base mutations. Prealbumin exhibited at least five amino acid substitutions per subunit but neither RBP nor prealbumin showed any differences in their NH_2-terminal sequences. Prealbumin of the two species exhibited partial identity on immunodiffusion analyses. This finding was corroborated by fluorescence titrations with use of prealbumin-specific monovalent antibody fragments. The RBP-binding region of prealbumin of the two species was apparently identical in spite of the fact that cynomolgus prealbumin obviously lacked four antigenic sites present on human prealbumin.

Vitamin A in human blood is predominantly bound to a specific transport protein, the retinol-binding protein (Kanai *et al.*, 1968; Peterson, 1969, 1971a). This vehicle for vitamin A which under physiological conditions is almost saturated with retinol binds to thyroxine-binding prealbumin (Kanai *et al.*, 1968; Peterson, 1969, 1971a). The complex formation of RBP[1] and prealbumin is highly specific and virtually all RBP in plasma is attached to prealbumin (Peterson, 1971b; Smith and Goodman, 1971). Recent results have shown that the interaction between RBP and prealbumin shields the RBP-bound retinol from the aquous surrounding (Peterson and Rask, 1971) thereby stabilizing the RBP–retinol interaction (Rask *et al.*, 1972). An obvious result of the formation of the prealbumin–RBP complex is the restricted loss of RBP from the blood through the glomeruli in the kidney (Vahlquist, 1972) since RBP in free form has a molecular weight of only 21,000 whereas that of the protein complex is 80,000 (Kanai *et al.*, 1968; Peterson, 1969, 1971a). Apart from the above-mentioned features of the prealbumin–RBP complex, nothing is as yet known about its detailed molecular function.

The highly specific protein complex of prealbumin and RBP, involving the ligands thyroxine and vitamin A, has so far only been detected in human blood. The evolutionary aspects of vitamin A transport in blood should be of considerable interest regarding the fact that a number of specific molecular interactions pertain to the system.

With the intention to study some evolutionary features of vitamin A plasma transport, in the present communication we have undertaken a comparative study of the prealbumin–RBP protein complexes isolated from human and cynomolgus plasma. Great similarities exist between the protein complexes of the two species although immunological differences between human and cynomolgus prealbumin are evident. The data suggest that prealbumin from the two species differs more than RBP.

Experimental Procedure

Materials

Cynomolgus Serum. Serum was obtained from cynomolgus monkeys (*Macaca irus*) (National Laboratory of Bacteriology, Stockholm). Serum was used immediately or stored at −23° until further processing.

Antisera. Antisera were raised in rabbits against purified C-RBP and C-prealbumin. The immunization procedure has been described (Peterson *et al.*, 1969). The antisera were shown to be specific by tests on Ouchterlony immunodiffusion and immunoelectrophoresis. Antisera against H-RBP and H-prealbumin were the same as used earlier (Vahlquist *et al.*, 1971). An antiserum against human serum proteins was purchased from Behringwerke AG (Marburg/Lahn).

Proteins. The prealbumin–RBP complex, prealbumin, and RBP were isolated from outdated human plasma as previously described (Rask *et al.*, 1971a).

Enzymes. Trypsin (code TRTPCK), pepsin (code PM BA), and carboxypeptidases A and B (code COADFP and COBC) were purchased from Worthington Biochemical Corporation (Freehold, N. J.).

Other Materials. Sepharose 4-B, Sephadex G-100 and G-200, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals AB, Uppsala, and prepared according to the instructions supplied by the manufacturer. Dansyl-amino acids and dansyl chloride were obtained from British Drug Houses, England. All other chemicals were reagent grade or of the highest quality available.

Methods

Isolation of Prealbumin and RBP from Serum of Cynomolgus Monkeys. RBP and prealbumin were isolated from a total

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[1] Abbreviations used are: RBP, retinol-binding protein; H, human; C, cynomolgus; Fab′ nomenclature for immunoglobulin fragments according to *Bull. W.H.O. 30*, 447 (1964).

of 16 l. of cynomolgus' serum. The purification procedure adopted was similar to that described for the human counterparts (Rask *et al.*, 1971a). Portions (2 l.) of serum were adjusted to 40% (w/v) with $(NH_4)_2SO_4$. The proteins remaining in the supernatant were separated on a column of DEAE-Sephadex, equilibrated with 0.015 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl. Elution was performed with a linear gradient of NaCl from 0.2 to 0.5 M. Fractions containing RBP and prealbumin were combined, concentrated, and subjected to further fractionation on a DEAE-Sephadex column equilibrated with 0.02 M Tris-HCl buffer (pH 7.2), containing 0.25 M NaCl. The column was developed with a linear gradient of NaCl from 0.25 to 0.5 M. This fractionation step resulted in the isolation of a highly purified prealbumin–RBP complex. Prealbumin and RBP were finally separated from each other by gel chromatography on a column of Sephadex G-100 equilibrated in 0.002 M Tris-HCl buffer (pH 8.0). The purified proteins were concentrated either by ultrafiltration or by lyophilization. Prior to lyophilization, the material was exhaustively dialyzed against distilled water.

The purity of C-RBP and C-prealbumin was assessed by polyacrylamide electrophoresis and by immunoelectrophoresis using antisera directed against human plasma proteins and against H-RBP, C-RBP, H-prealbumin, and C-prealbumin. All analyses revealed immunological homogeneity of the isolated proteins. Investigations by sedimentation-velocity and sedimentation-equilibrium ultracentrifugations indicated size homogeneity of both proteins (see below).

Preparation of Fab' Fragments against Prealbumin. Antibodies against H-prealbumin were isolated by immunoadsorption and monovalent Fab' fragments were prepared according to Nisonoff *et al.* (1960). The details of this procedure will be outlined elsewhere. The Fab' fragments inhibited the precipitation of prealbumin with an anti-prealbumin serum. The specificity of the Fab' fragments was also assessed by chromatography of ^{125}I-labeled Fab' fragments (Greenwood *et al.*, 1963) on a column of prealbumin-coupled Sepharose. Virtually all radioactivity (about 98%) was retained on the column. By these criteria it was concluded that the Fab' fragments were highly specific for prealbumin.

Since determinations by fluorescence titration of the reaction stoichiometry of Fab' fragments and an antigen require that all Fab' fragments are able to react with the protein, the following subfractionation of the specific Fab' fragments was accomplished.

To obtain monovalent antibodies reacting with C-prealbumin the Fab' fragments, directed against H-prealbumin, were passed over a Sepharose 4B immunoadsorbent on which C-prealbumin had been covalently attached. About 60% of the applied protein was retained on the column. The adsorbed Fab' fragments were eluted with 0.2 M glycine-HCl buffer (pH 2.9) and immediately titrated to pH 8 with 1 M Tris. Part of the nonadsorbed Fab' fragments was subsequently subjected to chromatography on a column of H-prealbumin immunosorbent. Virtually all Fab' fragments were retained, indicating that this population of the monovalent antibodies reacts with H-prealbumin but not with C-prealbumin. Monovalent antibodies reacting with the human and the cynomolgus prealbumin–RBP complexes were isolated in a similar way. The prealbumin-Sepharose immunoadsorbents were saturated with RBP prior to application of the Fab' fragments. The H-prealbumin immunoadsorbent retained about 65% of Fab' fragments whereas the C-prealbumin immunoadsorbent retained only about 35%. These retained Fab' fragments were eluted as described above and subsequently separated from eluted RBP by gel chromatography on Sephadex G-100 in 0.02 M Tris-HCl buffer (pH 8.0), containing 0.15 M NaCl.

Immunochemical Methods. Ouchterlony immunodiffusion analyses (Ouchterlony, 1962) were carried out as described elsewhere (Vahlquist *et al.*, 1971). Immunoelectrophoresis was performed according to the Scheidegger micromethod (Scheidegger, 1955). Quantitative precipitin analyses of C-RBP and H-RBP were performed with use of antisera directed either against H-RBP or C-RBP. Portions (200 μl) of these antisera, diluted 1:1, were incubated with various amounts of RBP. The mixtures were allowed to react for 2 hr at 25° followed by 48 hr at 4°. The precipitates were subsequently collected by centrifugation, repeatedly washed with ice-cold 0.15 M NaCl, dried, and dissolved in 0.5 M NaOH. The protein content was estimated by measuring the absorbance at 280 nm.

Gel Electrophoresis. Vertical polyacrylamide gel electrophoresis in slab form was carried out in the EC-474 apparatus (E-C Apparatus Corp., Philadelphia, Pa.). Electrophoresis was performed in 0.4 M Tris-glycine buffer (pH 8.9) with 4% Cyanogum 41 (E-C Apparatus Corp.) in the spacer gel and 8% in the running gel.

Starch gel electrophoresis in 8 M urea (Edelman and Poulik, 1961) was performed in formate buffer (pH 2.7). Gels of 1.8 mm thickness were run horizontally at a potential gradient of 25 V/cm for 75 min in a water-cooled apparatus for thin-layer electrophoresis (Desaga, Heidelberg). The details of this method have been outlined (Peterson and Berggård, 1971).

Measurements of Circular Dichroism. The circular dichroism spectrum of C-prealbumin was measured with a Jasco Model J-20 spectropolarimeter as outlined previously for H-prealbumin (Rask *et al.*, 1972). The reduced mean residue ellipticity $[\theta']$ was calculated using a mean residue weight of 110.

Amino Acid Analysis. Amino acid analyses were carried out according to Spackman *et al.* (1958). Protein samples (1–2 mg) were hydrolyzed in 6 N HCl at 110° for 24 or 72 hr (von Hofsten *et al.*, 1965). Half-cystine was determined as cysteic acid after performic acid oxidation. Tryptophan was estimated spectrophotometrically (Edelhoch, 1967).

Peptide Mapping. Peptide mapping of tryptic digests of RBP and prealbumin was performed on cellulose thin layers. Electrophoresis in pyridine–acetic acid buffer (pH 5.5) was followed by chromatography in the solvent described by Waley and Watson (1953). The details of this procedure have been outlined (Rask *et al.*, 1971a). The dried plates were sprayed either with ninhydrin (0.2% in acetone) or with phenanthraquinone (Yamada and Itano, 1966).

NH_2- and COOH-Terminal Analyses. NH_2-terminal sequence analysis of reduced and alkylated C-RBP and C-prealbumin were accomplished by Edman degradation plus dansylation (Gray, 1967). The liberated dansylamino acids were identified by two-dimensional chromatography on polyamide thin-layer sheets (Woods and Wang, 1967).

The COOH terminus of C-RBP was investigated with use of carboxypeptidases A and B. The procedure was according to Ambler as previously described (Rask *et al.*, 1971b).

Fluorescence Measurements. Fluorescence measurements were carried out using of an Aminco-Bowman and a Zeiss ZFM 4C spectrofluorometer as previously described (Peterson and Rask, 1971). All fluorescence measurements were performed at ambient temperature (23 ± 2°) in 0.02 M Tris-HCl buffer (pH 8.0), containing 0.15 M NaCl.

Association constants for the binding of RBP to prealbumin were measured by estimation of the quenching of the protein fluorescence on complex formation (Steiner *et al.*, 1966). The

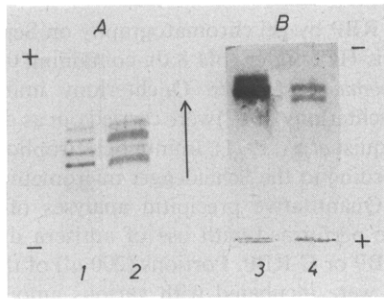

FIGURE 1: (A) Polyacrylamide gel electrophoresis in Tris-glycine buffer (pH 8.9) of H-RBP (1) and C-RBP (2). (B) Urea–starch gel electrophoresis at pH 2.7 of H-prealbumin (3) and C-prealbumin (4). The *arrow* indicates the direction of mobility.

quenching data were treated according to Scatchard (Scatchard, 1949).

The maximum number of specific Fab′-fragments which simultaneously could be bound to prealbumin was assessed by measurements of the resulting fluorescence quenching on interaction between Fab′ fragments and thyroxine-containing prealbumin or RBP–prealbumin complex. A detailed account of the experimental procedure will be given elsewhere.

Other Methods. Affinity chromatography of RBP on prealbumin-coupled Sepharose was performed as previously described (Vahlquist *et al.*, 1971). Analytical sedimentation-equilibrium ultracentrifugations were carried out as outlined elsewhere (Rask *et al.*, 1971a). Protein concentrations were usually measured by determining the optical density at 280 nm, relating the absorbance to the relevant extinction coefficients. Moisture in lyophilized samples of protein was determined by drying to constant weight at 100° and 0.05 mm of Hg.

Results

Some Physical and Chemical Characteristics of Prealbumin and RBP from Cynomolgus Monkeys. In Table I some physical and chemical properties of C-RBP and C-prealbumin are given. The corresponding data for the human counterparts are included in the table for comparison. It is evident from the table that by the methods used no easily discernible differences could be found. The molecular weight determination showed that C-prealbumin like H-prealbumin is composed of four noncovalently linked subunits of apparently identical size (Rask *et al.*, 1971a). On isoelectric focussing C-RBP displayed the same type of heterogeneity as previously recorded for H-RBP (Peterson and Berggård, 1971). The affinity of C-prealbumin for thyroxine and C-RBP exhibited similar association constants as were previously found for the human counterparts. The NH_2-terminal sequences for C- and H-RBP and for C- and H-prealbumin are apparently identical. It is noteworthy that arginine is the COOH-terminal residue in both C- and H-RBP. Previous results suggested that this residue is most important for the tertiary structure of H-RBP (Rask *et al.*, 1971b).

Gel Electrophoreses. RBP and prealbumin were subjected to polyacrylamide gel electrophoresis at pH 8.9. C-prealbumin and H-prealbumin appeared homogeneous and exhibited in each case a single band of similar if not identical mobility. As can be inferred from Figure 1A C-RBP as well as H-RBP gave rise to a total of six protein zones. Each band of H-RBP corresponded to a band of C-RBP although the relative distribution of material among the different zones varied between C-RBP and H-RBP. This variation is, however, noted for different preparations of the same protein. The interpretation of the electrophoretic heterogeneity of RBP has been discussed at length in a previous publication (Rask *et al.*, 1971b).

On urea–starch gel electrophoresis C-RBP and H-RBP appeared homogeneous and gave zones of identical mobility. C-prealbumin and H-prealbumin, however, displayed heterogeneity on urea–starch gels. It is evident from Figure 1B that both types of prealbumin showed two zones, probably representing tetramers and dimers (Gonzalez and Offord, 1971). A difference in mobility between C-prealbumin and H-prealbumin was clearly discernible. It may thus be concluded that C-RBP and H-RBP appeared identical on gel electrophoresis at neutral and acid pH, whereas a difference in electrophoretic mobility at acid pH for C-prealbumin and H-prealbumin was evident.

TABLE I: Some Physical and Chemical Characteristics of Prealbumin- and Retinol-Binding Protein.

	Retinol-Binding Protein		Prealbumin	
	Cynomolgus	Human[c]	Cynomolgus	Human[d]
Sedimentation coefficient, $s^0_{20,w}$ (S)	2.3	2.3		
Molecular weight				
By sedimentation equilibrium	20,500	21,000	58,000	62,000
By gel chromatography[a]	20,000	20,000	15,000	16,000
Isoelectric point (pH range)	4.4–4.8	4.4–4.8		
Molar extinction coefficient at 280 nm	40,000	39,100	87,100	85,800
Reduced mean residue ellipticity $[\theta']_{214\ nm}$ ((deg cm²)/dmole)			−5,400	−5,500
Association constants (M^{-1})				
Thyroxine[b]			1.6×10^7	1.2×10^7
RBP			2.0×10^7	1.6×10^7
NH_2-terminal sequence	Glu-Arg-Asx	Glu-Arg-Asx	Gly-Pro-	Gly-Pro-
COOH-terminal amino acid residue	Arg	Arg		

[a] Determined by gel chromatography on a Sephadex G-100 column equilibrated with 6 M guanidine hydrochloride. [b] Determined by equilibrium dialysis. [c] Data taken from Peterson (1971a), Peterson and Berggård (1971), and Rask *et al.* (1971b). [d] Data taken from Peterson (1971a), Peterson and Rask (1971), Rask *et al.* (1971a, 1972), and Nilsson and Peterson (1971).

TABLE II: Amino Acid Composition of Retinol-Binding Protein and Prealbumin from Cynomolgus Monkey.

	Retinol-Binding Protein			Prealbumin		
	Cynomolgus			Cynomolgus		
Amino Acid	Experimental[a]	To Nearest Integer	Human[g]	Experimental[a]	To Nearest Integer	Human[h]
	Residues/Molecule[b]					
Lysine	9.87	10	10	9.35	9	9
Histidine	2.00	2	2	4.27	4	5
Arginine	13.70	14	14	2.75	3	5
Aspartic acid	26.42	26	26	10.59	11	10
Threonine[c]	9.42	9	9	12.57	13	13
Serine[c]	11.37	11	11	12.24	12	11
Glutamic acid	19.39	19	18	14.13	14	14
Proline	6.02	6	7	8.21	8	7
Glycine	11.28	11	11	9.85	10	10
Alanine	13.98	14	14	13.12	13	13
Half-cystine[d]	5.13	5	5	1.00	1	1
Valine[e]	12.24	12	12	14.03	14	14
Methionine	3.18	3	4	1.00	1	1
Isoleucine[e]	5.05	5	4	4.33	4	6
Leucine	12.97	13	13	9.06	9	8
Tyrosine[c]	8.00	8	8	5.36	5	5
Phenylalanine	10.21	10	10	5.62	6	5
Tryptophan[f]	4.9	5	5	3.2	3	3

[a] Except where noted all figures are average values of one 24-hr and one 72-hr hydrolysis from four different determinations, of two separate preparations. [b] Calculations were based on the assumed presence of 2.00 residues of histidine per molecule of RBP and 1.00 residues of methionine per subunit molecule of prealbumin. [c] Values were obtained by extrapolation to zero hour of hydrolysis. [d] Determined after performic acid oxidation. [e] 72-hr hydrolysis value. [f] Determined spectrophotometrically. [g] Data taken from Rask *et al.* (1971b). [h] Data taken from Rask *et al.* (1971a).

Amino Acid Analyses and Peptide Mapping. The results of the amino acid analyses of C-RBP and C-prealbumin are summarized in Table II which for comparison also contains the corresponding data for the human counterparts. It is evident from the table that C-RBP and H-RBP exhibit great similarity in amino acid composition, the only apparent differences being one residue more of glutamic acid and isoleucine and one residue less of proline and methionine in C-RBP. C-prealbumin and H-prealbumin, however, display greater differences in amino acid composition, the C-prealbumin subunit containing one residue more of aspartic acid, serine, proline, leucine, and phenylalanine, two residues less of arginine and isoleucine and one residue less of histidine. It is thus apparent that C-prealbumin and H-prealbumin exhibit greater variation in amino acid composition than C-RBP and H-RBP.

In order to further elucidate the differences between C-RBP and H-RBP and C-prealbumin and H-prealbumin,

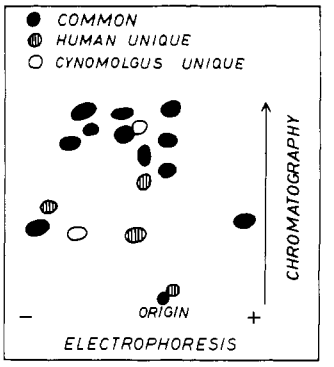

FIGURE 2: Schematic drawing representing a composite tryptic map of prealbumin from *Homo* and cynomolgus. *Filled* symbols represent peptides common to both species, stippled symbols represent peptides unique for *Homo*, and open symbols represent peptides unique for cynomolgus.

samples of each protein were separately digested with trypsin and subsequently subjected to peptide mapping. Approximately 25–30 ninhydrin-positive spots were detected for C-RBP and H-RBP, respectively. No reproducible difference was encountered between the two types of RBP.

Although the peptide maps of the two types of prealbumin were similar, reproducible differences were evident. Thus, H-prealbumin exhibited a somewhat greater number of ninhydrin-positive peptides (13–17) than C-prealbumin (11–15) (Figure 2). The number of phenanthraquinone positive peptides was also less for C-prealbumin which is in agreement with the observed difference in arginine content. The differences in number and locations of the peptides provide further evidence for a greater variability in prealbumin than in RBP between the two species.

Immunological Characteristics. The immunological relation of C-RBP and H-RBP was examined by Ouchterlony immunodiffusion analysis. The result obtained was compatible with complete identity of the two proteins irrespective of whether the antibodies were derived from an anti-C-RBP or an anti-H-RBP serum. However, as the Ouchterlony technique merely is a qualitative method, the immunological properties were further examined by the quantitative precipitin technique. The result, presented in Figure 3, demonstrates that both types of RBP reacted identically with an anti-H-RBP serum. Quantitatively the same result was obtained with use of an anti-C-RBP serum. C-RBP and H-RBP are thus indistinguishable by the immunological techniques employed.

Clear immunological differences were discernible on examination of C-prealbumin and H-prealbumin by Ouchterlony immunodiffusion analyses. Figure 4 shows that each protein contains, in addition to shared antigenic determinants, species-specific immunological reactivity as revealed by use of both anti-C- and anti-H-prealbumin sera.

The immunological differences between C-prealbumin and H-prealbumin were further examined by use of specific Fab′ fragments directed against H-prealbumin. By means of fluorescence quenching it was evident that C-prealbumin could maximally bind 8 Fab′ fragments simultaneously in contrast to the C-prealbumin–RBP complex which could only bind four Fab′ fragments (Figure 5). The corresponding numbers of Fab′ fragments maximally bound to the human counterparts were 12 and 8, respectively (unpublished results). This result seems to indicate that an antigenic region in C-prealbumin, which can maximally bind four Fab′ fragments, is

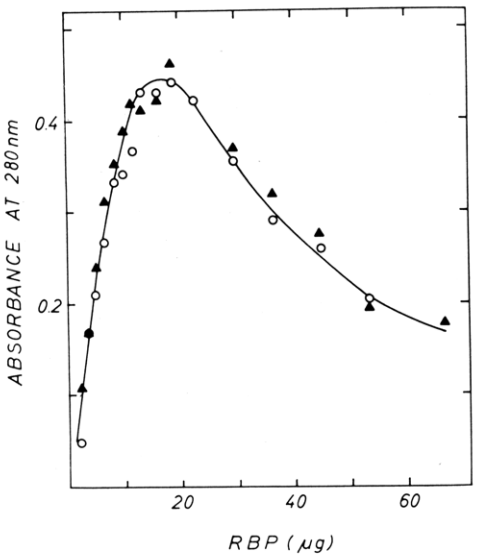

FIGURE 3: Quantitative precipitin curves of RBP obtained from *Homo* (▲) and cynomolgus (○) with use of an anti-H-RBP serum.

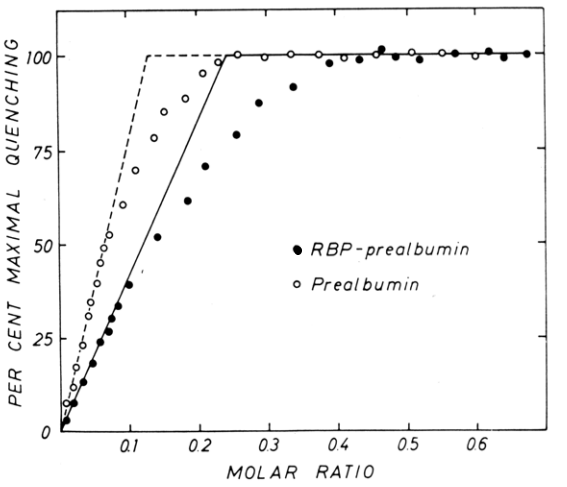

FIGURE 5: Stoichiometry of the reaction between cynomolgus prealbumin and specific Fab′ fragments. A constant concentration of Fab′ fragments (2×10^{-6} M) in 0.02 M Tris-HCl (pH 8.0), containing 0.15 M NaCl, was titrated with increasing amounts of prealbumin–thyroxine or prealbumin–RBP. The observed quenching of the Fab′ fragment tryptophyl fluorescence emission was corrected for the contribution of protein fluorescence from the added antigens. All values were corrected for dilution.

immunologically nonidentical with the corresponding region in H-prealbumin. However, it is evident that RBP is able to displace four Fab′ fragments from both types of prealbumin which is most easily explained assuming similar or identical RBP-binding sites on C-prealbumin and H-prealbumin.

Hybrid Prealbumin–RBP Complexes. When subjected to affinity chromatography on a H-prealbumin coupled Sepharose column C-RBP was completely retained, indicating that a strong interaction between the two proteins was operating. This result was further extended by measurements of the association constants of C-RBP and H-prealbumin as well as of H-RBP and C-prealbumin. The data from the fluorescence quenching experiments, given as a Scatchard plot, are presented in Figure 6. It is evident that both reacting systems, within experimental error, exhibit similar association constants which strongly suggests that the protein contact areas of the human and cynomolgus counterparts are very similar.

Discussion

Highly purified preparations of C-RBP and C-prealbumin exhibited characteristics very similar to those of their human counterparts when examined by physical–chemical methods. As expected, size and molecular weight are identical. RBP from the two species showed in addition identical charge characteristics both on acid and alkaline gel electrophoresis. Peptide maps and immunological investigations, both by qualitative and quantitative techniques, revealed no differences between the two proteins. The only apparent discrepancy encountered was by amino acid analysis. C-RBP exhibited one residue more of glutamic acid (or glutamine) and iso-

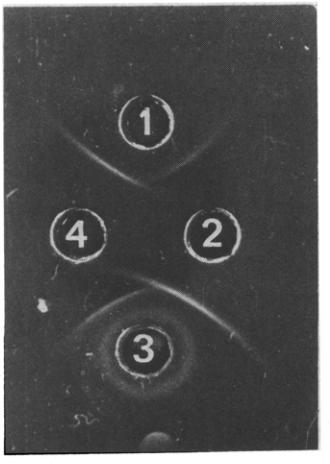

FIGURE 4: Ouchterlony immunodiffusion analysis of prealbumin from *Homo* (4) and cynomolgus (2) with use of antisera against H-RBP (1) and C-RBP (3).

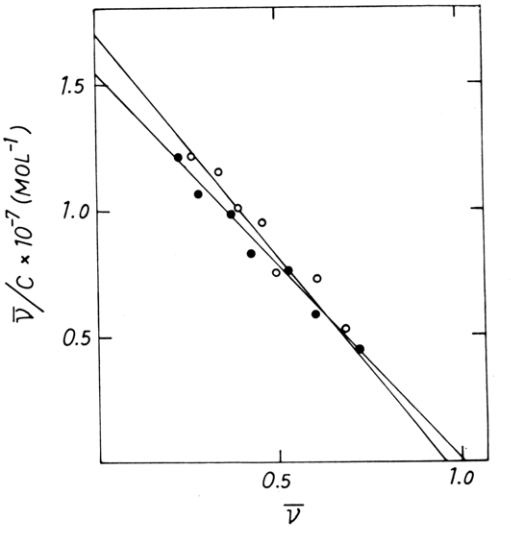

FIGURE 6: Binding of H-RBP to C-prealbumin (●) and of C-RBP to H-prealbumin (○) determined by fluorescence quenching in 0.02 M Tris-HCl buffer (pH 8.0), containing 0.15 M NaCl. The data were calculated using the equation of Scatchard (1949). $\bar{\nu}$ is the molar ratio of bound RBP to prealbumin and c the concentration of free RBP.

leucine and one residue less of proline and methionine. Although these differences were reproducible, consideration should be given to the fact that glutamic acid is present in relatively large amounts making the residue calculations of this amino acid liable to error. However, isoleucine, proline, and methionine are present in relatively small amounts giving a good accuracy of residue number determinations. Assuming a minimal number of substitutions in the codons it is likely that glutamine is substituted for proline and isoleucine for methionine, since both exchanges require only single base substitutions (Dayhoff, 1969). The resemblance between C-RBP and H-RBP is thus similar to that of cytochrome *c* but greater than for the hemoglobin chains of comparable species (Dayhoff, 1969). The restricted differences are, however, not unexpected considering that two features of RBP should conserve its structure; namely the interactions with vitamin A and prealbumin.

It is rewarding to find that the COOH-terminal amino acid of C-RBP, like for H-RBP, is arginine, since it has been noted that, for H-RBP, this residue is of importance for the three-dimensional structure of RBP (Rask *et al.*, 1971b). It was earlier shown that human serum contains one form of RBP which is devoid of vitamin A and the COOH-terminal arginine, and which cannot bind to prealbumin. On the basis of these findings it was suggested that liberation of the COOH-terminal residue occurred *in vivo* on releasing vitamin A from RBP to a target cell (Peterson, 1971b). This idea is now feasible for experimental test on cynomolgus monkeys.

Prealbumin isolated from cynomolgus plasma showed greater dissimilarities compared to its human counterpart than did RBP. Although prealbumin in the two species is composed of four apparently identical, noncovalently bound polypeptide chains, peptide maps revealed clearly discernible differences. The difference in number of ninhydrin-positive spots agrees well with the difference found in content of arginine, C-prealbumin having two residues less of arginine than H-prealbumin per subunit. The discrepancy in arginine content may also account for the different electrophoretic mobility noted on acid gel electrophoresis.

Immunological investigations of prealbumin corroborated the physical-chemical and chemical analyses. Common as well as species-specific determinants were present on the protein. With use of fluorescence quenching titration it has been shown that H-prealbumin can bind maximally 12 anti-H-prealbumin Fab′ fragments (unpublished results). Since it is well established that prealbumin is composed of 4 identical polypeptide chains (Rask *et al.*, 1971a; Gonzalez and Offord, 1971; Blake *et al.*, 1971; Branch *et al.*, 1971; Morgan *et al.*, 1971), and since hemoglobin, with subunits of similar size as prealbumin, can bind 12 Fab′ fragments simultaneously, three on each polypeptide chain (Noble *et al.*, 1969), it is tentative that the prealbumin sunbunit also binds three Fab′ fragments. It may thus be inferred that C-prealbumin, which binds a total of eight Fab′ fragments directed against H-prealbumin, shares two antigenic regions per subunit with H-prealbumin, whereas the third site obviously is species specific. RBP, when interacting, displaces a total of four Fab′ fragments both from H-prealbumin and C-prealbumin. This may indicate that one Fab′ fragment is displaced per prealbumin subunit and that one of the shared antigenic regions is located close to or at the RBP-binding site. The similarity of the RBP-binding sites on H-prealbumin and C-prealbumin is also indicated by the fact that the affinity between H-prealbumin and C-RBP and *vice versa* is identical within experimental error with that estimated for the homologous complex formation. This conclusion is consistent with the idea that a high degree of constancy during evolution must be preferred in those stretches of the polypeptide chain directly involved in the binding of the two proteins, since amino acid substitutions in one of the proteins may require a complementary substitution in the other protein, not to interfere with complex formation. It may hence be suggested that most amino acid substitutions in prealbumin and RBP occur in parts of the polypeptide chains not directly involved in the binding of the two proteins.

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Susceptibility of Paramyosin to Proteolysis and the Relationship to Regions of Different Stability†

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ABSTRACT: Similar proteolytic-resistant cores were obtained by digestion of paramyosin from the adductor muscles of the clam, *Mercenaria mercenaria*, with a wide variety of enzymes (chymotrypsin, nagarse, pepsin, Pronase, and trypsin). Detailed studies were made on paramyosin pepsin-resistant core (PPC) and paramyosin trypsin-resistant core (PTC). Both cores were from the N-terminal portion of paramyosin and Cys was the only N-terminal amino acid found. Both cores were completely helical by circular dichroism and fluorescence, and the high intrinsic viscosities of 120 cm^3/g at pH 7.4 and 157 cm^3/g at pH 2.0 were consistent with the helical structure. Reversibility of denaturation (5 M guanidine hydrochloride (Gdn·HCl), pH 2.0, 55°) as well as sodium dodecyl sulfate gel electrophoresis indicated that each core consisted of two intact polypeptide chains. Determination of molecular weight by three methods gave the same average value of 140,000 for both cores. It is concluded that PPC and PTC are identical except for minor differences at their C-terminal ends. Proteins similar to PPC or PTC could not be detected in extracts of adductor muscle of *M. mercenaria*. Regions in the paramyosin molecule differ in their stability to heat and Gdn·HCl. The C-terminal region is least stable and the N-terminal region is most stable. The C-terminal one-third of paramyosin lost its helical structure upon heating at pH 2 (transition temperature 57°) whereas PPC and PTC (from the N-terminal two-thirds of paramyosin) were stable over the region 20–70°. Denaturation by Gdn·HCl at pH 2 and 20° occurred in two major stages at 5 and 7 M Gdn·HCl. From changes in fluorescence, it was estimated that the loss of helical structure in 5 M Gdn·HCl was 64% for paramyosin and 34% for PPC and PTC; again, the C-terminal segment seemed least stable. In 7 M Gdn·HCl both paramyosin and the cores behaved as completely random coils. Conversion from the intermediate stage of denaturation in 5 M Gdn·HCl at 20° to completely random coil could also be effected by heat. The transition temperature of 46° was the same for paramyosin, PPC, and PTC. This is in accord with the other denaturation studies and supports the conclusion that the most stable region of paramyosin is at the N-terminal portion.

Paramyosin is one of the major contractile proteins extractable from adductor muscle of molluscs (Hodge, 1952; Bailey, 1957; Kominz *et al.*, 1957). The contractile units of molluscan muscle consist of thick and thin filaments (Hanson and Lowry, 1959; Philpott *et al.*, 1960), and paramyosin is the major component of the thick filaments (Hanson and Lowry, 1961). More recently, Szent-Györgyi *et al.* (1971) have demonstrated that the paramyosin molecules align to form a bipolar core of the thick filament which is covered by a surface layer of myosin. This paramyosin core seems to modify the interaction of myosin with actin of the thin filament and thereby to have a specific regulatory role in maintenance of muscle tension. The paramyosin molecule appears to be well suited to such a role for it is a large molecule of 220,000 atomic mass units that is rod shaped, 1330 Å long by 20 Å diameter, and consists of two intertwined α-helical chains (Lowey *et al.*, 1963).

Our laboratory is engaged in studies of the detailed structure of the paramyosin molecule both to help clarify the specific role of this molecule in the molluscan thick muscle filament and as a model system for more general studies of the stability of the helical structure and of interaction between helical rodlets. In the course of these studies it was observed that paramyosin from *Mercenaria mercenaria* upon digestion by any one of several proteolytic enzymes yielded a large segment of the original molecule resistant to further digestion by any of these enzymes. Proteolytic digestion of paramyosin from *Pinna nobilis* had been reported (Bailey and DeMilstein, 1964; Bailey *et al.*, 1964) to yield a large segment of 102,000 atomic mass units that was nearly two-thirds of the mass of the original molecule. These results are similar to those observed for paramyosin from *M. mercenaria*. The principal difference was that the segment from *P. nobilis* was largely nonhelical (35% helical) whereas the segment isolated from *M. mercenaria* has been found to be completely helical. Because this large helical segment permitted interesting chemical and physical comparisons with the parent molecule, the size and shape of this segment and its location within the parent molecule were determined. In addition, some of the comparative studies, notably stability to Gdn·HCl[1] and heat, will be reported, and regions of different stability will be

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[1] Abbreviations used are: TNBS, trinitrobenzenesulfonic acid; Gdn·HCl, guanidine hydrochloride; PPC, paramyosin pepsin-resistant core; PTC, paramyosin trypsin-resistant core; T_{tr}, transition temperature.