PARTIAL CHARACTERIZATION OF MOLECULAR SPECIES OF RETINOL-BINDING PROTEIN FOUND IN TUBULAR PROTEINURIA DUE TO CHRONIC CADMIUM POISONING IN THE RABBIT

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Summary A study was conducted to characterize the molecular species of retinol-binding protein (RBP) isolated from the urine of rabbits chronically poisoned with cadmium. The RBP species, identical with regard to both molecular size (approximately 20,000) and immuno-reactivity, were separated into four fractions by means of polyacrylamide gel electrophoresis (PAGE), which yielded two holo-RBP (H2 and H1) and two apo-RBP (A2 and A1) species. The urinary excretion ratio of these fractions (H2: H1: A2: A1) was found to be about 70: 6: 21: 3. No distinct difference of amino acid composition between holo- and apo-RBP was observed. An additional species of apo-RBP (designated An) was also isolated from the aged holo-RBP (H2) by isoelectric focusing in gel.

Using the separated molecular species of rabbit RBP as well as of human RBP, their interactions with human prealbumin (PA) were examined both by human PA-Sepharose affinity chromatography, and by gel filtration on Sephadex G-100 after in vitro incubation of the RBP with human PA. Purified rabbit holo-RBP exhibited almost the same binding ability to human PA as did human RBP. Retinol within RBP molecule enhanced the affinity to PA, presumably through the change of tertiary structure, although the presence of retinol was not essential for the protein-protein interaction.

It has been well established that retinol (vitamin A) is transported in plasma by a specific protein, retinol-binding protein or RBP, which has been isolated and

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Abbreviations: RBP, retinol-binding protein; PA, prealbumin; PAGE: polyacrylamide gel electrophoresis.
characterized in many species including man (1), rat (2), monkey (3), pig (4), cow (5) and chicken (6). RBP has a molecular weight of close to 20,000, a single binding site for one molecule of retinol and migrates in the α-region on electrophoresis. Under physiological condition, RBP circulates as a tight 1:1 molar complex with prealbumin or PA, which has a molecular weight of about 55,000 (6–8). The association of RBP with PA increases the stability of the retinol-RBP complex (9, 10), and prevents the ready filtration of RBP through the renal glomerulus.

Of interest is the fact that urinary excretion of RBP is observed in patients with tubular proteinuria due to chronic cadmium intoxication in Sweden (11) and with “Itai-Itai” disease2 in the limited districts of Japan (12, 13). The urine of those patients contains relatively large amounts of RBP and provides a good source for purification of the protein with excellent yields. Recently, we have reported that the rabbit given long-term administration of cadmium chloride shows urinary excretion of RBP, and we have described the isolation and partial characterization of rabbit holo-RBP (14). During the course of the study, we have also observed several other forms of immunoreactive RBP in the urine, which have now been isolated and partially characterized as described in the present paper.

EXPERIMENTAL

Isolation of molecular species of rabbit and human RBP

Four molecular species of rabbit RBP were isolated from the pooled urine by a sequence of procedures which included: Gel filtration on Sephadex G-100, chromatography on DEAE- and SP-Sephadex, and finally preparative isoelectric focusing in gel as illustrated in Fig. 1. Specific details for collection of the urine and column chromatography were reported in the previous paper (14). Two forms of holo-RBP (designated H2 and H1) and two of apo-RBP (designated A2 and A1) were distinguishable by relative mobility on polyacrylamide gel electrophoresis (PAGE). The holo-RBP species designated H2 corresponds to the protein characterized as described in the previous report (14).

Molecular species of human RBP were simultaneously purified from the urine in patients with “Itai-Itai” disease by virtually similar procedures to those of the rabbit proteins as described above.

Preparation of antisera and immunological methods.

A monospecific anti-rabbit RBP was raised in a goat using holo-RBP (H2) as an antigen as reported in the previous paper (14). Anti-human RBP was prepared against purified human urinary apo-RBP (A2) as outlined previously (2).

Cross immunoreactivity among RBP molecular species was examined by double immunodiffusion using the method of OUCHTERLONY (15). Concentrations

2 “Ouch-Ouch” disease or chronic entero-oste-o-nephropathy cadmium.
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Urine 10.3 liter (Lyophilized protein: 17 g)

Sephadex G100

Peak A (Complex)  Peak B (Free)

DEAE-Sephadex A-50

Peak C  Peak D

Sephadex G-100  Sephadex G-100

Peak E  Peak E'

SP-Sephadex C-50  SP-Sephadex C-50

Peak G  Peak H

Apo-RBP (A2)  Holo-RBP (H2)

Peak G'  Peak H'

Apo-RBP (A1)  Holo-RBP (H1)

Fig. 1. Schematic diagram of isolation procedures of RBP molecular species in the urine of rabbit chronically poisoned with cadmium.

of apo-RBP as well as holo-RBP in eluates obtained by column chromatography were determined by single radial immunodiffusion technique according to the method of Mancini et al. (16).

**Gel electrophoresis**

In order to obtain relative mobility on PAGE, the purified RBP molecular species were simultaneously subjected to gel columns, and electrophoresis was done under the same condition according to the method of Davis (17).

Analytical and preparative isoelectric focusing in gel were performed as described by Righetti and Drysdale (18). Carrier ampholites (Ampholine, pH 4 to 6, LKB Produkter, Sweden) was used. Acrylamide and N,N'-methylene bis-acrylamide (Nakarai Chemicals Ltd., Kyoto) were re-crystallized by acetone before use. In an analytical experiment, 40 μg of the protein, dissolved in 50 μl of 2% Ampholine and 6% sucrose, was applied to two gel columns (3 x 105 mm) containing 2% Ampholine, 5% acrylamide and 0.2% N,N'-methylene bis-acrylamide (20 μg protein per column). Focusing was performed on an apparatus (Medical Research Apparatus, Corp., Boston) at a constant voltage of 400 V for 17 hr at 0°C, using a cooling system (Neslab Instruments, Inc., Portsmouth, N.H.). After focusing, the one gel was immediately treated with 10% trichloroacetic acid for one hour and stained with 0.25% Coomassie Brilliant Blue R (Tokyo Chemical Industry Co., Tokyo) in 45.4% methanol and 9.2% acetic acid for 5 min, and destained overnight. The other gel was sliced into 5 mm
wide segments, which were dispersed in 0.2 ml of distilled water overnight, and then pH of those gel eluates were measured. In a preparative experiment, 700 µg of protein sample was applied to a column (12 × 150 mm). Specific details are indicated in the legend to Fig. 3.

**Amino acid analysis**

Amino acid compositions of the molecular species of rabbit and human RBP were determined simultaneously as described in the previous paper (14). Because of the very limited amounts of purified rabbit RBP molecular species available, analyses were carried out on only one sample each (0.3–0.4 mg), subjected to acid hydrolysis for 24 hr.

**Extraction and reconstitution of retinol with RBP**

Extraction of retinol from holo-RBP was carried out in the presence of 0.5 M NaCl according to the method of Futterman and Heller (19). About 90% of the ligand was removed by a single extraction with ethanol from the RBP as judged by the ratio of absorbance at 330 nm to 280 nm.

Retinol was reconstituted with native or chemically extracted apo-RBP as described by Heller and Horwitz (20). In a typical experiment, 89 nmoles of retinol (Sigma Chemical Co., St. Louis) in 1 µl of ethanol was added to 17.8 nmoles of apo-RBP³ in 0.5 ml of 0.02 M potassium phosphate buffer, pH 7.4 containing 0.2 M NaCl. Immediately after addition, the contents were mixed vigorously, and incubated at 4°C in the dark for one hour.

**Binding of RBP with PA**

Binding studies were performed in two ways: [1] Affinity chromatography of RBP on a column of human PA co-valently coupled to Sepharose; “Affinity chromatography” method, and [2] Gel filtration on Sephadex G-100 after in vitro incubation of human PA with RBP; “Gel filtration” method.

³ E₁%₁ calves at 280 nm of RBP and PA were reported to be 16 (1) and 14 (7), respectively, and these values were used for molar calculation in this study.
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[2] Gel filtration on Sephadex G-100 was used for separation of complexed RBP with PA from the free RBP. In a typical experiment, equimolar RBP to PA (17.8 nmoles) was incubated at 4°C for 20 min after mixing, and then applied onto a column of Sephadex G-100 equilibrated with 0.02 M potassium phosphate buffer, pH 7.4 containing 0.2 M NaCl. Specific details for illustrative examples are also indicated in the legend to Fig. 4. With the gel filtration procedures, a small amount of blue dextran polymer with molecular weight of $2 \times 10^6$ was usually added prior to chromatography in order to obtain the void volume; $V_v$.

Other Procedures

Protein concentration was determined by both the absorbance at 280 nm, and the method of Lowry et al. (22) using bovine serum albumin as a standard. Protein-bound retinol was estimated either by the relative intensity of fluorescence with excitation at 330 nm and emission at 460 nm, respectively or by the UV absorbance at 330 nm.

RESULTS

Partial physico-chemical properties of molecular species of rabbit RBP

Four molecular species of rabbit RBP were finally isolated, each exhibiting a single band on individual runs of PAGE. The four species had different relative mobilities on electrophoresis and isoelectric points as indicated in Table 1. In general, the apo-species migrated distinctly faster than the respective holo-proteins. The isoelectric point between A2 and H1 became indistinguishable, although the two proteins were still well separated from each other on PAGE. Rabbit RBP species displayed slower electrophoretic mobility and higher isoelectric points than the respective species of human RBP.

The four molecular species of rabbit RBP (H2, A2, H1 and A1) were found to be indistinguishable with regard to their elution patterns on gel filtration on Sephadex G-100. The molecular weight of species H2 was determined previously to be approximately 20,000 by SDS-containing PAGE method (14). Moreover, all these four proteins gave reactions of complete immunological identity by double immunodiffusion as indicated in Fig. 2. Using single radial immunodiffusion assay, percent of these RBP species found in the urine was calculated to be H2, 70%; A2, 21; H1, 6; A1, 3, respectively.

The results of amino acid analyses of the molecular species of both rabbit and human RBP are given in residues per molecule obtained simultaneously after 24 hr of acid hydrolysis (Table 1). Two RBP species in rabbit; H1 and A1 were not investigated because of the very limited supply of the proteins. Only small differences were observed between the RBP species, which were within the error limits of the methods as used.

An additional apo-RBP species (designated An) was artificially produced
Table 1. Partial physico-chemical properties of molecular species of urinary
RBP in rabbit in comparison with those of human RBP.

|                     | Rabbit RBP | Human RBP |
|---------------------|------------|-----------|
|                     | H2         | A2        | H1        | A1        |
| R<sub>f</sub> on PAGE | 0.54       | 0.59      | 0.61      | 0.65      |
| pI                  | 5.1        | 4.9       | 4.9       | 4.8       |
|                     |            | 0.55      | 0.58      | 0.63      | 0.67      |
| Amino acid composition: residues per molecule (nearest integer) |                 |                 |            |            |
| Lysine              | 10         | 10        | —<sup>a</sup> | —<sup>a</sup> | 10         | 10        | 10        | 10        |
| Histidine           | 4          | 4         | —<sup>a</sup> | —<sup>a</sup> | 2          | 2         | 2         | 2         |
| Arginine            | 14         | 13        | —<sup>a</sup> | —<sup>a</sup> | 14         | 13        | 13        | 13        |
| Aspartic acid       | 29         | 28        | —<sup>a</sup> | —<sup>a</sup> | 25         | 24        | 24        | 24        |
| Threonine           | 7          | 7         | —<sup>a</sup> | —<sup>a</sup> | 9          | 8         | 8         | 9         |
| Serine              | 11         | 10        | —<sup>a</sup> | —<sup>a</sup> | 11         | 10        | 10        | 10        |
| Glutamic acid       | 15         | 16        | —<sup>a</sup> | —<sup>a</sup> | 19         | 16        | 18        | 18        |
| Proline             | 6          | 6         | —<sup>a</sup> | —<sup>a</sup> | 7          | 5         | 6         | 6         |
| Glycine             | 11         | 11        | —<sup>a</sup> | —<sup>a</sup> | 13         | 12        | 12        | 12        |
| Alanine             | 12         | 13        | —<sup>a</sup> | —<sup>a</sup> | 14         | 13        | 13        | 13        |
| Half-cystine        | 6          | 6         | —<sup>a</sup> | —<sup>a</sup> | 6          | 6         | 6         | 6         |
| Valine              | 14         | 13        | —<sup>a</sup> | —<sup>a</sup> | 12         | 11        | 11        | 10        |
| Methionine          | 4          | 4         | —<sup>a</sup> | —<sup>a</sup> | 4          | 4         | 4         | 4         |
| Isoleucine          | 3          | 3         | —<sup>a</sup> | —<sup>a</sup> | 3          | 3         | 3         | 3         |
| Leucine             | 12         | 12        | —<sup>a</sup> | —<sup>a</sup> | 12         | 12        | 12        | 12        |
| Tyrosine            | 7          | 7         | —<sup>a</sup> | —<sup>a</sup> | 8          | 8         | 8         | 8         |
| Phenylalanine       | 12         | 12        | —<sup>a</sup> | —<sup>a</sup> | 10         | 10        | 10        | 10        |
| Tryptophan          | 3          | 3         | —<sup>a</sup> | —<sup>a</sup> | 3          | 4         | 3         | 4         |

<sup>a</sup> Not determined due to the limited amounts of RBP specimens.

Fig. 2. Ouchterlony double immunodiffusion of the individual molecular species of rabbit RBP; H2, A2, H1 and A1, when tested against a monospecific goat anti-rabbit holo-RBP; H2 (designated Anti-H2).
Fig. 3. Elution pattern of the aged rabbit holo-RBP (H2) on preparative isoelectric focusing in gel. The protein (700 μg), dissolved in 0.6 ml of 2% Ampholine and 6% sucrose, was applied to a gel column (12×150 mm) containing 2% Ampholine, 5% acrylamide and 0.2% N,N'-methylene bis-acrylamide. Focusing was carried out at a constant voltage of 400 V for 21 hr at 0°C. After focusing, the gel was sliced into 35 segments (2 or 5 mm wide), which were dispersed in 2 ml of distilled water for 16 hr. The pH, relative intensity of fluorescence specific for protein-bound retinol, and concentrations of immunoreactive RBP in the eluates were determined. “Nascent” apo-RBP or An was well separated from the holo-RBP; H2.

Comparative studies on binding of human PA with RBP molecular species in rabbit and human

Studies were conducted to examine the chemical and functional properties of the RBP molecular species, in particular, the interaction of the proteins with human PA, using both “Affinity chromatography” and “Gel filtration” method as described in EXPERIMENTAL.

Holo-RBP obtained from either human or rabbit displayed a tight complex
Fig. 4. Binding of human PA to human or rabbit holo-RBP by "Affinity chromatography" (left panel), and "Gel filtration" method (right panel).

(A): Human holo-RBP; H2 (1.8 mg in 2 ml of the starting buffer) was applied onto a human PA-Sepharose column (1.4×4.9 cm). Fractions of 2 ml were collected at a flow rate of 20 ml per hour. The procedure for washing and elution was performed as described in EXPERIMENTAL. The arrow denoted the start of distilled water, pH 10.

(B): Human holo-RBP; H2 (total O.D._280 nm=0.62, O.D._330 nm=0.48) and human PA (total O.D._280 nm=1.43) were dissolved in 0.5 ml of 0.02 M potassium phosphate buffer, pH 7.4 containing 0.2 M NaCl. After incubation for 20 min at 4°C, the mixture was applied to a column of Sephadex G-100 (1×79 cm) equilibrated with the same buffer. Fractions of 1 ml were collected at a flow rate of 5 ml per hour. The center of the protein-bound retinol peak was found to be fraction 29 (V_e/V_o=1.21).

(C): Rabbit holo-RBP; H2 (0.9 mg in 2 ml of the starting buffer) was applied to the same human PA-Sepharose column as indicated in the panel (A).

(D): Rabbit holo-RBP; H2 (total O.D._280 nm=0.54, O.D._330 nm=0.42) and human PA (total O.D._280 nm=1.24) were mixed and applied to the same column of Sephadex G-100 as described in the panel (B).

formation with human PA. Most of both human and rabbit holo-RBP (H2) were eluted as peak II by human PA-Sepharose affinity chromatography as shown in Fig. 4 (A) and (C). By "Gel filtration" method, both human and rabbit H2
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species exhibited a protein-protein complex with human PA as shown in Fig. 4 (B) and (D). The percent distribution of immunoreactive RBP species found either in peak II by “Affinity chromatography” or in the complexed fraction by the “Gel filtration” method are indicated in Table 2 (the upper column). Both human and rabbit holo-RBP species H1 also revealed virtually the same results to those of H2, using both binding techniques (Table 2).

Table 2. Binding ability of human PA to rabbit or human RBP molecular species by (A): “Affinity chromatography,” and by (B): “Gel filtration” method.

| (A) | “Affinity chromatography” | (B) | “Gel filtration” method |
|-----|---------------------------|-----|-------------------------|
|     | Peak I        | Peak II | Complexed | Free |
| (1) | Holo-RBP      |          |           |      |
| human | H2         | 14%     | 86%       | 85%  |
|       | H1         | 11%     | 89%       | 87%  |
| rabbit | H2       | 11%     | 89%       | 87%  |
|       | H1         | 13%     | 87%       |      |
| (2) | Extracted (apo) RBP |          | 25%       | 75%  |
| human | apo-H2   |          | 67%       | 33%  |
|       | +retinol  |          | 17%       | 83%  |
| rabbit | apo-H2 |          | 72%       | 28%  |
|       | +retinol  |          |           |      |
| (3) | Apo-RBP    |          |           |      |
| human | A2        | 20%     | 80%       |      |
|       | A2+retinol | 28%     | 72%       |      |
|       | A1        | 17%     | 83%       | 56%  |
|       | A1+retinol| 20%     | 80%       | 86%  |
| rabbit | A2+retinol|          | 50%       | 50%  |

b Not examined due to the limited supply of RBP molecular species.

Binding of human PA with chemically extracted (apo) RBP, obtained originally from both human and rabbit holo-RBP, H2, was examined by the “Gel filtration” method as shown in the middle column of Table 2. For this particular experiment, we used the retinol-extracted (apo) human RBP (total O.D_{280 nm} = 0.62) and rabbit protein (total O.D_{350 nm} = 0.46), still containing about 10% of holo-RBP. After incubation of these proteins with equimolar human PA, most of the apo-RBP was eluted as uncomplexed or free forms by gel filtration on Sephadex G-100 (75% in human and 83% in rabbit, respectively). Protein-bound retinol, observed in the complexed fraction, was considered to be derived from the unextracted portion of holo-RBP in the preparation.

Both human and rabbit retinol-extracted RBP or apo-H2 were incubated with five-fold molar excess of retinol as mentioned in EXPERIMENTAL. The retinol-reconstituted holo-RBP used for this experiment was as follows: total O.D_{280/330 nm}
=0.66/0.50 for human and 0.46/0.35 for rabbit preparation, respectively. By incubation with equimolar human PA, most of the retinol-reconstituted RBP (67% in human and 72% in rabbit protein) were eluted as complexed fraction on Sephadex G-100 column, as clearly shown in Table 2 (the middle column).

Small portions of the retinol-extracted and -reconstituted RBP in both human and rabbit were subjected to analytical PAGE to study changes in electrophoretic mobility. In human holo-RBP, the major band of the retinol-extracted or apo-H2 migrated in similar region to that of native apo-RBP; A2 ($R_f=0.58$). On the other hand, the retinol-reconstituted RBP displayed the virtually same position as that of original holo-RBP; H2 ($R_f=0.55$).

In contrast, the rabbit apo-H2 or extracted RBP gave a relative mobility of 0.57 on PAGE, which was consistent with that of "nascent" apo-RBP or A2 as described previously (different from the relative mobility of native apo-RBP; A2). After reconstitution of the protein with retinol, electrophoretic mobility returned to that of original holo-RBP; H2.

Several binding experiments between human PA and native apo-RBP found in urine of both human and rabbit were also performed, using either native or retinol-reconstituted apo-RBP, as indicated at the bottom of Table 2.

By the "Affinity chromatography" method, human apo-RBP, A2 and A1 (0.4 to 0.7 mg of protein used) were eluted as peak II, regardless of reconstitution with retinol (about 80% of apo-RBP can bind with PA). On the other hand, "Gel filtration" method revealed a reduced binding of apo-RBP (A1) with human PA, which was significantly enhanced after reconstitution with retinol (from 56% to 86% in complexed fraction). In addition, both retinol-reconstituted human apo-RBP; A2 and A1 showed virtually similar electrophoretic mobilities to those of native holo-RBP; H2 and H1, respectively.

Only one typical binding experiment of rabbit apo-RBP (A2), reconstituted with retinol, was performed, mainly due to the very limited supply of the protein (Table 2). Prior to incubation with five-fold molar excess of retinol, a clear solution of apo-RBP (total O.D. $_{280\text{nm}}=0.44$) was obtained after centrifugation at 9,000 $g$ for 10 min, and then the reconstituted protein was applied to the same column of Sephadex G-100 together with equimolar human PA. Fifty percent of the RBP revealed a complex formation with human PA, exhibiting fluorescence specific for protein-bound retinol. However, the other half of the protein did not display any binding with either retinol or human PA.

**DISCUSSION**

The present study clearly demonstrates that at least four molecular species of retinol-binding protein or RBP (designated H2, A2, H1 and A1) can be isolated from the urine of rabbits chronically poisoned with cadmium, being virtually similar to those of human RBP found in tubular proteinuria or "Itai-Itai" disease (12, 13).
The urinary excretion ratio of these RBP species (H2: H1: A2: A1) obtained from the rabbit was found to be 70: 6: 21: 3 by single radial immunodiffusion assay. Hence, the isolation of H1 and A1 species proved to be somewhat difficult in comparison with the H2 fraction. With regard to molecular size (approximately 20,000) and immunological reactivity (Fig. 2), however, the three rabbit RBP species (H1, A2 and A1) finally isolated were indistinguishable from holo-RBP (H2), which was characterized in some details as reported previously (14). No distinct difference of amino acid composition between H2 and A2 was also observed (Table 1). Taken together with both clinical (23) and experimental studies (24, 25) on the renal handling of RBP, these findings strongly suggest that the molecular species of rabbit urinary RBP are originated from a common plasma holo-RBP, presumably identical with the H2 species obtained from the urine.

Despite similar physico-chemical properties between rabbit and human RBP (molecular weight, electrophoretic mobility, isoelectric point, amino acid composition, binding ability with retinol and prealbumin or PA), the two proteins are immunologically distinct. In addition, the greater part of urinary RBP molecular species in rabbit consists of holo-RBP (H2+H1; 76%), whereas predominantly apo-RBP (about 70–85%) can be found in the urine of patients with “Itai-Itai” disease (12, 26). This difference between rabbit and human RBP remains to be elucidated. Of interest is the observation on “nascent” apo-RBP or An, isolated from rabbit holo-RBP, H2 (Fig. 3). As already reported in the previous paper (14), the fresh preparation of the H2 protein showes no microheterogeneity on PAGE, in sharp contrast to the greater conversions to H1 and A2 in even fresh preparations of human plasma RBP (H2) (27).

During preservation for 4 months (freezing and thawing), the aged rabbit H2 revealed a very faint, and slightly faster band on PAGE (Rf=0.57) without fluorescence (apo-form). Similar result was obtained by holo-RBP in chicken plasma (6). Moreover, studies of electrophoretic mobility on PAGE of both the retinol-extracted H2 and retinol-reconstituted An provide good evidence that H2 and An (not A2) are interconvertible. It is possible that human holo-RBP converts easily to respective apo-protein during excretion through the urinary tract as well as preservation and isolation of the proteins. On the other hand, rabbit holo-RBP is excreted in the urine without greater production of apo-species (As to development of rabbit A2, the other mechanism or further structural change(s) must be considered for explanation).

Moreover, RBP circulates as a protein-protein complex with PA. In the present paper, we adopted two methods for RBP-PA interaction: “Affinity chromatography” and “Gel filtration” technique as described in EXPERIMENTAL. As clearly shown in Fig. 4, rabbit holo-RBP was found to bind with human PA. The finding supports the previous observations on qualitative bindings of human PA with monkey (3), porcine (4), bovine (5) and rat RBP4, of human RBP with

4 KANDA, Y. et al. presented at the 10th International Congress of Nutrition, Kyoto, 1975.
rat PA, and of chicken PA with bovine RBP (5), respectively. Hence, it is now conceivable that certain important structural or conformational similarity of binding site with PA may exist widely among the mammalian species, regardless of distinct structural differences in the antigenic site.

It has been reported by Raz et al. (27), and Rask et al. (28) that the presence of retinol bound to the RBP molecule is not a prerequisite for the formation of a protein-protein complex of RBP with PA. As shown in Table 2, however, retinol-reconstituted apo-RBP (native and previously retinol-extracted or apo-H2) markedly enhanced the binding affinity of RBP to human PA by "Gel filtration" method.

In contrast, rabbit urinary apo-RBP (A2) displayed the reduced binding affinity to human PA (Table 2). About a half of the protein, which is able to bind with retinol, showed a protein-protein complex with PA, when the protein was previously incubated with five-fold molar excess of retinol. Taken together with the interconvertibility between rabbit holo-RBP (H2) and apo-RBP (An), the rabbit A2 is assumed to be distinct from human A2. The reason why rabbit A2 could not bind efficiently with human PA remains to be explained. One of the most serious problems, however, would be partial denaturation of the rabbit A2 protein. In fact, the A2 preparation becomes turbid and has some difficulties for solubilization in a buffer solution. For the particular experiment as indicated in Table 2, we used the supernatant immediately after centrifugation without a long-standing solubilization.

It is intriguing us to speculate a physiological role of individual RBP molecular species as initially proposed by Rask et al. (28). However, the hypothesis remains controversial from the following two major reasons: 1) Questions about C-terminal residue of holo-RBP as arginine, and 2) Possibility of artificial denaturation of Rask’s apo-RBP preparation: the apo-RBP was exclusively eluted as peak I materials without any affinity to PA on the affinity chromatography (no study on reconstitution of the peak I materials with excess retinol is available). In contrast, most of urinary apo-RBP from patients with “Itai-Itai” disease can bind with both retinol and PA.

In any event, the present study on the molecular species of rabbit urinary RBP strongly suggests that major component in glomerular filtrate is holo-RBP, in particular H2 species, which will be derived from a small, but metabolically active pool of uncomplexed RBP in the plasma (25). The possibility of specific change(s) in primary structure of RBP (i.e. C-terminal residue) for delivering retinol (vitamin A) to cell membrane of the target tissue still remains to be elucidated. However, RBP is assumed to be one of the fine molecules, since retinol within the protein brings about conformational change with regard to binding affinity to PA, probably due to changes in net charge of the molecule. Thus, the physico-chemical

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5 Leucine as C-terminal residue has been reported (29), and the other residue also detected by KANDA, Y. et al. (personal communication).
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property behaves beneficial for RBP as one of transport proteins in the body.

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