



PURIFICATION AND PROPERTIES OF PORCINE ALLANTOIC FLUID RETINOL-BINDING PROTEIN

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ABSTRACT

Retinol-binding protein (RBP) was purified from Day 60 porcine allantoic fluid by a combination of diethylaminoethyl cellulose, G-100 Sephadex, G-50 Sephadex, Phenyl-Sepharose, and Reactive Green 19-dye-agarose chromatography. The yield was 1 to 2 mg of RBP, which generated a single M_r ~20,000 band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and up to four isoelectric variants (isoelectric variants 1 to 4) after two-dimensional PAGE (2D-PAGE). The protein cross-reacted with antiserum raised against human RBP. When incubated with [³H]retinol and subjected to G-100 Sephadex chromatography, [³H]retinol coeluted with the protein. These results indicate that the purified protein is an RBP. When purified RBP was subjected to native 2D-PAGE, six forms of RBP were observed. Three native forms were fluorescent, and three were not fluorescent, suggesting that these forms were RBP with and without retinol, respectively. Denaturing 2D-PAGE analysis of each native form of RBP suggested that two of the nonfluorescent and two of the fluorescent native forms of RBP corresponded to isoelectric variant 1 on denaturing 2D-PAGE, whereas the other fluorescent and nonfluorescent forms corresponded to isoelectric variant 2. The incubation of RBP with 50 μ M retinol enhanced the amount of both isoelectric variants present as fluorescent RBP, but uptake by isoelectric variant 1 was greater than that by isoelectric variant 2. These data indicate that RBP can be purified from porcine allantoic fluid and suggest that the isoelectric variants may differ in their affinity for retinol.

INTRODUCTION

Retinol is required for the normal development of many tissues (1,2). In adult plasma, retinol is bound to retinol-binding protein (RBP), which prevents the abnormal interaction of retinol with tissues (3) and also protects retinol from oxidation and other chemical modifications (4). RBP is an M_r ~20,000, pI 5.5 protein (5) that is a member of a larger family of binding proteins (6-9).

Plasma RBP is synthesized primarily in the liver, which is also the main site of retinol storage (10). However, other tissues also secrete RBP (11-13). During pregnancy in the pig, both the endometrium (14-16) and the conceptus (17,18) secrete RBP. Endometrial and conceptus secretion of RBP is thought to be involved in the transport of retinol to the developing fetus. The neonatal pig uterus also secretes RBP, which may serve to increase local concentrations of retinol during uterine development (19). Purified RBP would allow studies of the function of this protein in retinol delivery to the fetus during pregnancy and to other tissues that require retinol for normal function.

Both the endometrium and the conceptus during pregnancy and the uterus during the neonatal period secrete RBP consisting of isoelectric and molecular-weight variants. The molecular origin of these variants is not known and the properties of the variants have not been extensively studied. Clawitter et al. (15) and Stallings-Mann et al. (16) concluded that isoelectric variants present in uterine flushings do not differ in their ability to bind

retinol and probably have no physiologic relevance. The isoelectric variants were identified by their elution from an ion-exchange high-performance liquid chromatography (HPLC) column. However, the relationship between the forms of RBP observed by the use of HPLC and those observed on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was not established. Because the modifications of RBP that result in the presence of the isoelectric variants may influence retinol binding and therefore may have an influence on retinol delivery to target tissues, a reexamination of the binding of retinol by porcine RBP was undertaken. The objectives of the following experiments were to purify RBP from Day 60 porcine allantoic fluid and to characterize the resulting isoelectric variants for their ability to bind retinol.

MATERIALS AND METHODS

Purification of RBP From Day 60 Allantoic Fluid. The methods used in handling animals in this experiment meet the requirements of our Institutional Animal Care and Use Committee. Porcine allantoic fluid was collected at slaughter from conceptuses of several sows on Day 60 of gestation (Day 0 = first day of estrus), pooled, and frozen at -20°C until processed further. To purify RBP from allantoic fluid, the allantoic fluid (1 to 2 l) was dialyzed against three changes of 8 l of 10 mM Tris (pH 8.2)–0.02% NaN_3 (Buffer A). The dialyzed allantoic fluid was loaded onto a 2×13 cm diethylaminoethyl-cellulose (DEAE) column (DE52; Sigma Chemical Co., St. Louis, MO) previously equilibrated in Buffer A, and then, proteins were eluted with a 500-ml 0 to 0.3 M NaCl gradient in Buffer A. The presence of protein and RBP in fractions was determined by absorbance at 280 nm and fluorescence (Perkin Elmer LS50; excitation wavelength, 330 nm; emission wavelength, 470 nm), respectively (RBP could also be detected with a handheld UV light [365 nm]). Fractions containing RBP were combined, dialyzed against Buffer A, and concentrated by the use of a 5-ml DEAE column eluted with 10 ml of Buffer A + 1 M NaCl. The concentrated proteins were then chromatographed on a 2×80 cm G-100 Sephadex column equilibrated in Buffer A + 0.33 M NaCl. Fractions containing RBP were combined, dialyzed against Buffer A, concentrated with DEAE, and chromatographed on a 2×80 cm G-50 Sephadex column equilibrated in Buffer A + 0.33 M NaCl. Fractions containing RBP were combined, made to 35% saturation with solid ammonium sulfate, and loaded onto a 1.5×4 cm Phenyl-Sepharose-CL4B column equilibrated in Buffer A + 1 M ammonium sulfate. The column was eluted with a 200-ml, 1 to 0 M ammonium sulfate gradient in Buffer A followed by 50 ml of Buffer A. Fractions containing RBP were combined, dialyzed in Buffer A, concentrated on DEAE, redialyzed against Buffer A, and then passed through a 0.5×10 cm Reactive Green 19-agarose column. The purity of the resultant protein was confirmed by sodium dodecyl sulfate–PAGE (SDS-PAGE), followed by staining with coomassie and also silver. The concentration of protein after each stage in the purification was determined by use of the method of Lowry et al. (20) with bovine serum albumin (BSA) as standard. The amount of retinol present after each stage in the purification was determined by use of a modification of the method of Selvaraj and Sushella (21), as described by Vallet et al. (19)

Generation of Specific Antiserum to Porcine RBP. Rabbits were immunized with 100 μg of RBP in Freund's complete adjuvant, followed at 2-wk intervals with two booster immunizations of 50 μg in Freund's incomplete adjuvant. Two weeks after the final immunization, serum was collected. The specificity of the antiserum for purified RBP, RBP in allantoic fluid, and RBP in serum was demonstrated by immunoblotting.

Characterization of Purified RBP. The immunologic similarity of the purified RBP to authentic RBP was demonstrated by immunoblotting with anti-human RBP antiserum (Dako Corp., Carpinteria, CA). The binding of retinol by the purified RBP was demon-

strated by the incubation of 100 μg of the protein with 2 μCi of [11, 12- $^3\text{H}(\text{N})$]retinol (37.1 Ci/mmol; NEN DuPont Co., Wilmington, DE) in Buffer A for 2 hr at room temperature, followed by chromatography on a 1.5×80 cm G-100 Sephadex column equilibrated with Buffer A + 0.33 M NaCl. Fluorescence and radioactivity in each fraction were determined.

The native forms of RBP were characterized by the use of native 2D-PAGE. Fluorescent forms were identified with a handheld UV light (365 nm). To determine which native forms of RBP corresponded to the isoelectric variants of RBP observed by denaturing 2D-PAGE, the protein spots on a stained native 2D-PAGE gel were cut from the gel, incubated at room temperature overnight in denaturing 2D-PAGE gel-loading buffer, and then subjected to denaturing 2D-PAGE. To determine whether the nonfluorescent native forms of RBP were capable of binding retinol, 50 μg of RBP was combined with either no retinol or 50 μM retinol and incubated for 2 hr ($n = 3$ replicates). Samples were then subjected to native 2D-PAGE. The effect of exogenous retinol on the amounts of fluorescent (retinol containing) and nonfluorescent native forms of RBP were confirmed by the use of laser densitometry (Ultrascan XL; Pharmacia LKB, Piscataway, NJ) after gels were stained with coomassie. Finally, to examine the effect of native protein conformation on the migration of RBP during gel electrophoresis, 100 μg of RBP was subjected to electrophoresis by the use of a procedure identical to that for native 2D-PAGE, except that 8 M urea was included in the sample buffers and gels.

Electrophoresis and Immunoblotting. SDS-PAGE was performed as described by Buhi et al. (22). The 2D-PAGE was performed as described by Roberts et al. (23), except that Triton X-100 was used instead of Nonidet P-40. Native isoelectric focusing gels were prepared by use of the following modifications to the procedure for denaturing 2D-PAGE: 1) urea and detergent were deleted; 2) only the 5-7 ampholine was used; 3) the top surface of the gel was not overlaid during polymerization; 4) samples for native 2D-PAGE were dissolved in 5 mM K_2CO_3 -20% glycerol (native 2D-PAGE loading buffer); 5) fast green in 5 mM K_2CO_3 -10% glycerol (10 μl) was used as tracking dye; and 6) the electrophoresis apparatus was cooled to 4° C. For the second dimension, a 15% acrylamide running gel and a 4.5% acrylamide stacking gel were prepared as for denaturing 2D-PAGE, except that no SDS was added to the gels and the gels were overlaid with distilled water. The isoelectric focusing tube gels were incubated for 10 min in 10 mM Tris (pH 6.8) and then fixed to the top of the stacking gel with a solution of 1% low-melting-temperature agarose in 10 mM Tris (pH 6.8)-0.001% bromophenol blue-0.001% xylene cyanol (tracking dyes). Chamber buffer (20 mM Tris-190 mM glycine) was added, and the samples were electrophoresed at 20 mamps until the tracking dyes migrated through the stacking gel and then were electrophoresed either at 25 mamps for 4 to 5 hr or 7 mamps overnight. The bottom buffer chamber was cooled to 4° C during electrophoresis to prevent the degradation of proteins.

For immunoblotting, gels were blotted onto nylon-supported nitrocellulose (Midwest Scientific, Valley Park, MO; 24). To detect RBP, blots were incubated in 3% BSA in Tween buffer (0.1% BSA, 140 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , 0.5% Tween-20) overnight at 4° C. The blots were then incubated for 2 hr at room temperature with a 1:2,500 dilution of anti-RBP antiserum, washed four times with Tween buffer (20-min incubation per wash), incubated with a 1:100,000 dilution of anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase antibody conjugate for 2 hr at room temperature, and then washed four times with Tween buffer. The blots were then rinsed with 0.9% saline. Specific binding was detected with 3,3'-diaminobenzidine tetrachloride (one 10-mg tablet [Sigma Chemical Co.] to 100 ml of 0.9% saline-0.03% H_2O_2).

Statistical Analysis. To evaluate the effect of exogenous retinol on the saturation level of the isoelectric variants of RBP, densitometric measurements were made for native forms u and v combined, and native forms w and x combined and separate density measurements were made for native forms y and z. For isoelectric variant 1 from denaturing 2D-PAGE, percent saturation was calculated as the densitometric measurement of native forms u and v combined (retinol bound, isoelectric variant 1), divided by the densitometric measurements of native forms u and v combined plus w and x combined (retinol-bound forms plus retinol-free forms to obtain the total amount of isoelectric variant 1), multiplied by 100. For isoelectric variant 2 from denaturing 2D-PAGE, percent saturation was the densitometric measurement of native form y (retinol-bound form, isoelectric variant 2), divided by the densitometric measurement of native forms y and z combined (retinol-bound form plus retinol-free form to obtain the total amount of isoelectric variant 2), multiplied by 100. This is valid because results indicated that forms u, v, w, and x are native forms of isoelectric variant 1 and y and z are native forms of isoelectric variant 2 (see Results). The percent saturation data were then subjected to analysis of variance (ANOVA); Statistical Analysis System; SAS Institute, Cary, NC), with and without ARCSINE transformation, by the use of a model that included the effects of retinol treatment, isoelectric variant, and their interaction.

RESULTS

Purification of RBP From Day 60 Allantoic Fluid. Representative chromatograms from each step in the purification of RBP from allantoic fluid are illustrated in Figure 1. With the exception of Phenyl-Sepharose, the purification could be easily monitored with the known fluorescence of retinol when it is bound to RBP. Phenyl-Sepharose chromatography appears to partially separate fluorescent RBP (RBP-retinol) from nonfluorescent RBP because appreciable amounts of protein identified as RBP by SDS-PAGE and immunoblotting (data not shown) continued to elute from the column after the fluorescent RBP. Two-dimensional PAGE analysis of purified protein followed by coomassie staining revealed two major isoelectric variants (Figure 2a). Coomassie and silver (not shown) staining indicated that contamination of the purified RBP with other proteins was very low. Table 1 illustrates the amount of protein and retinol in the RBP fraction from each step in the purification. By use of the results obtained, assuming molecular weights of 287 and 20,000 for retinol and RBP and assuming a one-to-one ratio of retinol to RBP, the purified RBP is 40% saturated with retinol. Approximately 1 to 2 mg of RBP can be purified from 1 to 2 l of Day 60 allantoic fluid.

Characterization of Porcine Allantoic Fluid RBP. Two-dimensional PAGE followed by immunoblotting of purified RBP with anti-human RBP antiserum revealed two major and two minor isoelectric variants of purified protein (Figure 2b). When the purified RBP was incubated with [³H]retinol and subsequently chromatographed, a peak of [³H]retinol cochromatographed with a peak in fluorescence, indicating that the purified RBP is capable of binding retinol (Figure 1f).

Generation of Antisera to Porcine RBP. Immunoblots of purified RBP (Figure 2c), allantoic fluid proteins (Figure 3a), and serum proteins (Figure 3c) with antiserum generated against porcine allantoic fluid RBP are illustrated. The antiserum generated to porcine allantoic fluid RBP revealed the same four isoelectric variants as that observed with anti-human RBP antiserum (Figure 2b). The two major isoelectric variants 1 and 2 were easily visible on the immunoblot of allantoic fluid proteins, but only isoelectric variant 1 of RBP was detected when serum was immunoblotted.

Native 2D-PAGE of Purified RBP. Six forms of RBP were observed when purified RBP was subjected to native 2D-PAGE followed by coomassie staining, and they are

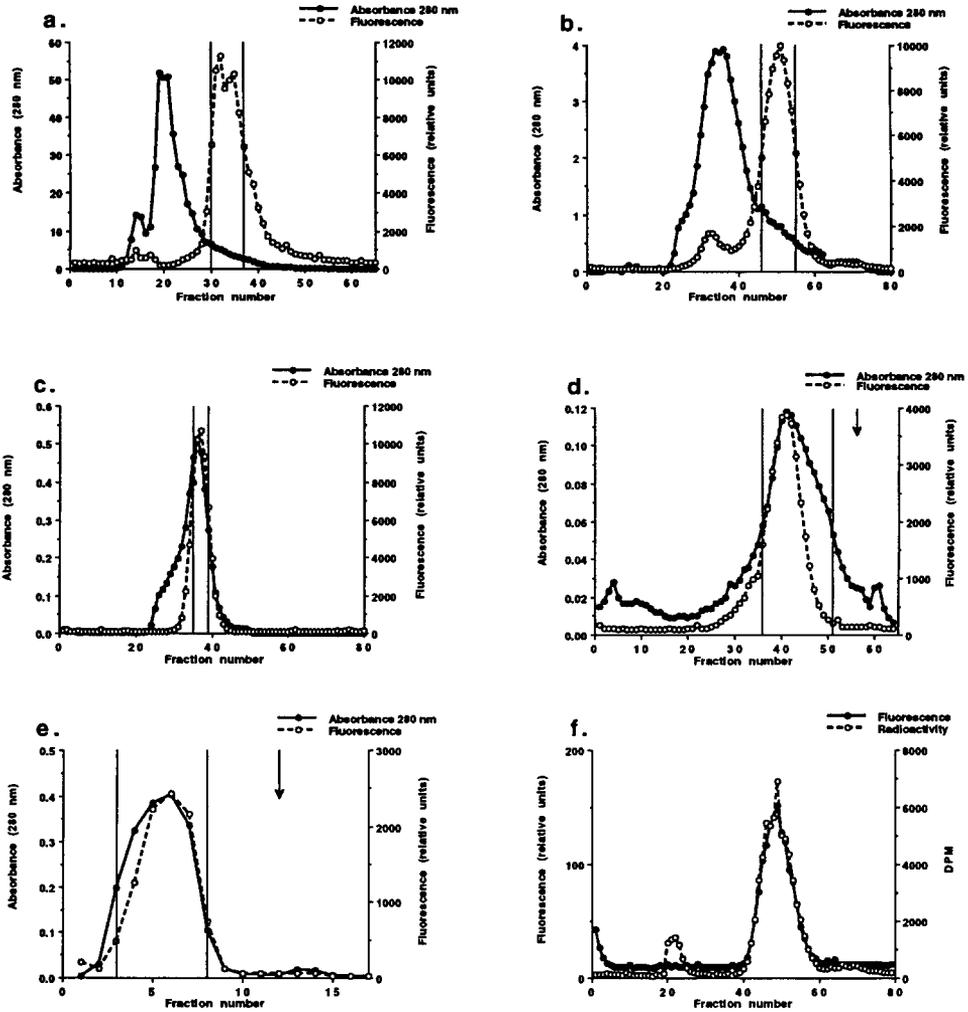


Figure 1. Chromatograms showing absorbance at 280 nm and fluorescence in each fraction from each step of RBP purification from allantoic fluid and fluorescence and radioactivity in each fraction from chromatography of RBP mixed with [³H]retinol are illustrated. (a) DEAE cellulose chromatography of Day 60 allantoic fluid; (b) G-100 Sephadex chromatography of the RBP fraction from DEAE chromatography; (c) G-50 Sephadex chromatography of the RBP fraction from G-100 chromatography; (d) Phenyl-Sepharose chromatography of the RBP fraction from G-50 Sephadex chromatography (arrow marks the end of the gradient and the changeover to 10 mM Tris [pH 8.2]); (e) Reactive Green 19 chromatography of the RBP fraction from Phenyl-Sepharose chromatography (arrow marks change over to 10 mM Tris-1 M NaCl [pH 8.2]); (f) G-100 Sephadex chromatography of purified RBP (100 μg) incubated with 2 μCi of [³H]retinol for 2 hr before application to the column. Vertical lines in graphs indicate fractions that were pooled and applied to the next step.

illustrated in Figure 4a. Three of the six forms were fluorescent (native forms u, v, and y; not shown) and therefore contained retinol. When each spot was cut from the gel and subjected to denaturing 2D-PAGE, native forms u, v, w, and x (lettering from Figure 4a) from native 2D-PAGE of RBP all contained predominantly isoelectric variant 1 and minor amounts of isoelectric variants 2 and 3 from denaturing 2D-PAGE (numbering from Figure 2b). Native forms y and z from native 2D-PAGE (lettering from Figure 4a) both contained predominantly isoelectric variant 2 and minor amounts of isoelectric variant 3 (numbering from Figure 2b) after denaturing 2D-PAGE (Figure 5). These results suggest

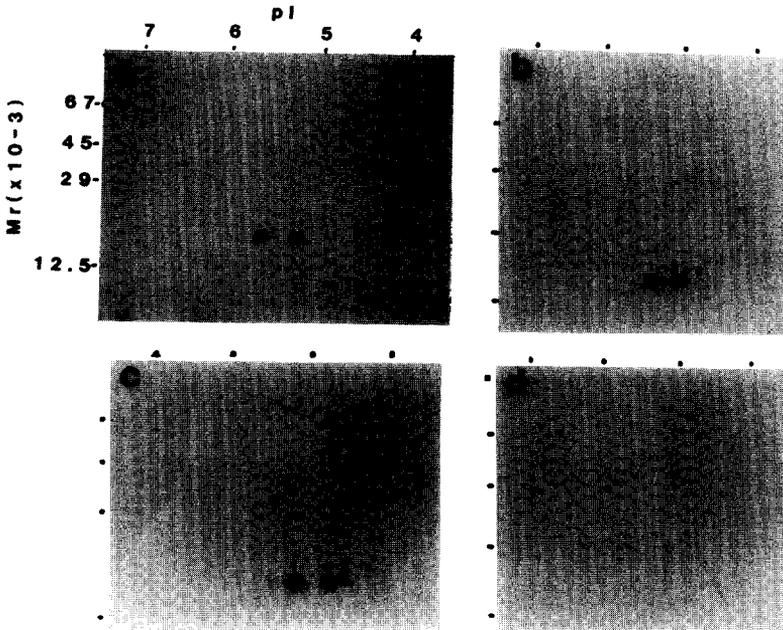


Figure 2. Representative 2D-PAGE gel of purified RBP (10 μ g) stained with coomassie (a) and 2D-PAGE immunoblots of purified RBP (10 μ g) detected with anti-human RBP antiserum (b), antiserum generated to purified porcine RBP (c), or normal rabbit serum (d).

that native forms u, v, w, and x are different conformations of isoelectric variant 1 from denaturing 2D-PAGE; u and v are retinol-bound forms, and w and x are retinol-free forms of this isoelectric variant. Likewise, native forms y and z are different conformations of isoelectric variant 2 from denaturing 2D-PAGE; y is the retinol-bound form and z is the retinol-free form. In the presence of urea (Figure 4b), purified RBP migrated predominantly as two isoelectric variants (numbered 1 and 2). Each isoelectric variant appeared to be composed of at least two molecular-weight variants (lettered a and b for each isoelectric variant; Figure 4b).

The interaction of isoelectric variants 1 and 2 from denaturing 2D-PAGE with retinol was tested by incubating RBP with exogenous retinol (Figure 6). The amounts of isoforms u and v combined, w and x combined and y and z were measured by densitometry of the coomassie-stained gels. Saturation of each isoelectric variant was then calculated as the

TABLE I. THE AMOUNTS OF PROTEIN, RETINOL, AND SPECIFIC ACTIVITY, YIELD, AND FOLD PURIFICATION OF RBP AT EACH STEP IN THE PURIFICATION PROCEDURE

Purification Step	Protein (mg)	Retinol (μ g)	Specific Activity (ng/mg)	Yield (%) ^a	Fold Purification ^a
D60 Allantoic fluid	4,000	161	40		
Diethylaminoethyl chromatography	208	49	236	30	5.9
G-100 Sephadex	21	49	2338	29	58.5
G-50 Sephadex	8.75	31	3580	19	89.5
Phenyl-Sepharose	2.125	10.5	4932	7	123.3
Reactive Green 19	1.60	9.3	5815	6	145.4

^a Yield and fold purification were calculated with retinol in the RBP fraction measured by a modification of the method of Selvaraj and Sushella (21) and assumes no loss of retinol from RBP during purification.

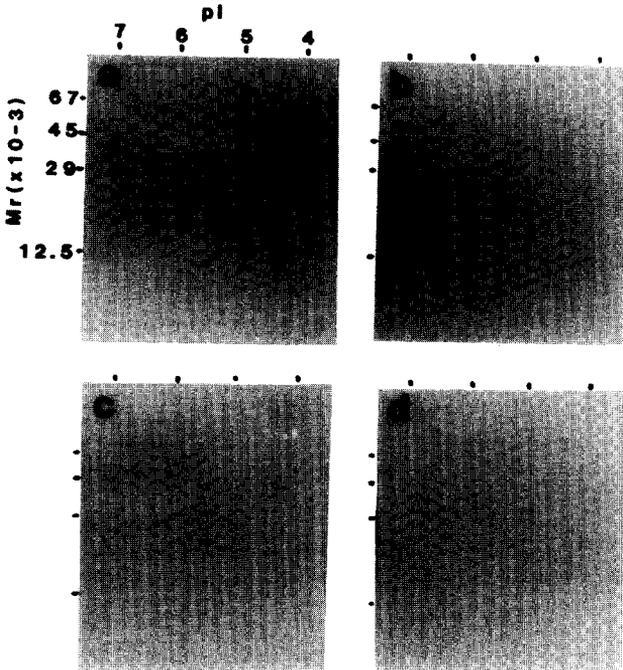


Figure 3. Representative 2D-PAGE immunoblots of allantoic fluid protein or pig serum detected with the antiserum that was generated to purified porcine RBP (a and c) or normal rabbit serum (b and d). Gels were loaded on the basis of RBP content (1 μ g) measured by radioimmunoassay (28).

density of the retinol-bound forms (u and v for isoelectric variant 1 and y for isoelectric variant 2), divided by the total density for that variant (u, v, w, and x for isoelectric variant 1 and y and z for isoelectric variant 2), multiplied by 100. A variant by retinol treatment interaction was present ($P = 0.066$ without transformation; $P = 0.074$ after ARCSINE transformation) and resulted from a greater increase in saturation for isoelectric variant 1

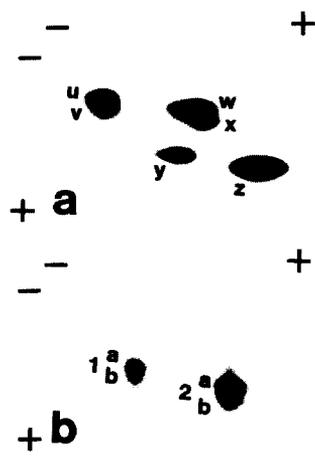


Figure 4. Representative 2D-PAGE gels showing the native variants (lettered u to z) of purified RBP (a) and the variants (lettered a and b for each numbered isoelectric variant) of purified RBP in the presence of 8 M urea (b). Forms u, v, and y in panel (a) were fluorescent (not shown).

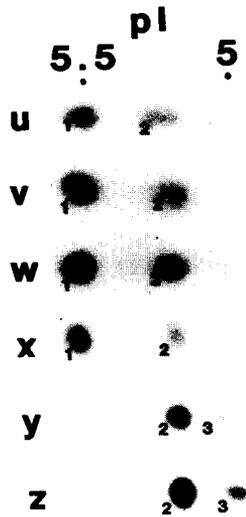


Figure 5. Isoforms of RBP resulting from 2D-PAGE of RBP forms u to z from native 2D-PAGE (Figure 4a). Gels were stained with coomassie. Numbers correspond to the numbering of spots indicated in Figure 2b.

(33.8% increased to 65.4%) after retinol treatment compared with isoelectric variant 2 (35.9% increased to 49.7%; SE for each mean from ANOVA was 5.1%). These results suggest that isoelectric variants 1 and 2 from denaturing 2D-PAGE may differ in their affinity for retinol.

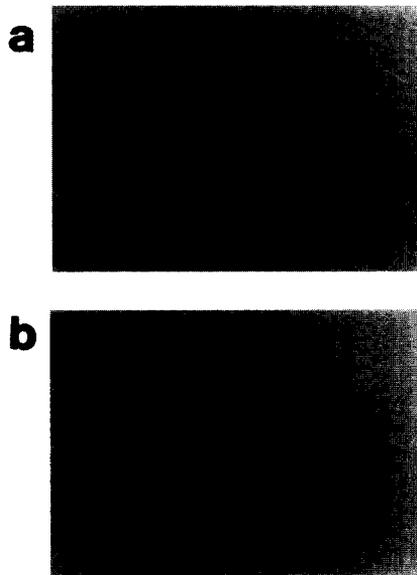


Figure 6. Native 2D-PAGE gels stained with coomassie after samples (50 μg) of RBP were incubated with either distilled water (a) or 50 μm of retinol (b). Retinol treatment increased the percentage of isoelectric variant 1 migrating as forms u and v (retinol-bound forms of isoelectric variant 1). Retinol treatment had less effect on the percentages of isoelectric variant 2 migrating as form y (retinol-bound form isoelectric variant 2; see Results).

DISCUSSION

These results are the first to indicate that RBP can be purified in useful quantities from porcine allantoic fluid and that RBP from allantoic fluid differs from RBP obtained from porcine serum in that isoelectric variants of RBP are present in allantoic fluid. This report is also the first to indicate that many native forms of porcine RBP exist and that they appear to be the result of different conformations of the same isoelectric variant from denaturing 2D-PAGE. Results suggest that the isoelectric variants of porcine RBP may differ in their affinity for retinol. The changes in RBP that generate the isoelectric variants may therefore have physiologic relevance.

The purification of RBP from 1 to 2 l of allantoic fluid resulted in 1 to 2 mg of purified RBP that was approximately 40% saturated with retinol. This agrees well with the densitometry data, which suggest that the saturation is between 30 and 40%. The low saturation rate of RBP from allantoic fluid is not necessarily unexpected, given the known function of the allantoic sac as a waste and storage compartment. It is likely that RBP that has lost its retinol through normal physiologic processes within the fetus will be deposited in the allantoic sac. Excess retinol-RBP may be delivered to the allantoic sac in the same way, in a manner similar to that hypothesized for uteroferrin (25).

Allantoic fluid RBP consisted of several isoelectric variants on denaturing 2D-PAGE and immunoblotting, which is similar to RBP secreted by the conceptus (17), endometrium (15), and neonatal uterus (19) in culture. Adult serum RBP did not contain isoelectric variants and therefore differed from RBP from the other sources. This observation differs from those of others, who observed variants of human serum RBP, especially in serum after prolonged storage (26). However, after a period of more than 1 year, porcine serum kept at -20°C still did not contain isoelectric variants of RBP, whereas recently collected allantoic fluid always contains isoelectric variants (not shown).

Analysis of native forms of RBP was undertaken to determine whether there were forms of RBP that were incapable of binding retinol. Results of native 2D-PAGE analysis of allantoic fluid RBP suggest that many native forms of RBP are present in the purified preparation. Several experiments were performed to determine the origin of these various forms of RBP. When native forms u, v, w, and x were cut from a stained native 2D-PAGE gel and subjected to denaturing 2D-PAGE analysis, the predominant isoelectric variant was isoelectric variant 1 from denaturing 2D-PAGE, along with minor amounts of isoelectric variants 2 and 3. Similar treatment of native forms y and z resulted in predominantly isoelectric variant 2 from denaturing 2D-PAGE, with minor amounts of isoelectric variant 3. These results suggest that native forms u, v, w, and x are each native forms of isoelectric variant 1 and that native forms y and z are each native forms of isoelectric variant 2 from denaturing 2D-PAGE. The presence of minor amounts of the other isoelectric variants in each spot may be caused by incomplete separation of the native forms of RBP (i.e., minor contamination of each native form with the other forms) or by spontaneous conversion of the less acidic isoelectric variants to the more acidic isoelectric variants after native 2D-PAGE separation. Similar interconversion of isoforms of RBP was reported by Raz et al. (26). The conversion of the six native forms of RBP on native 2D-PAGE to two isoelectric variants with two M_r variants at each pI when the gels were run in the presence of urea again suggests that many of the native forms of RBP are different native conformations of the same isoelectric variant.

Results of incubating RBP with retinol suggest that native forms w and x can be converted to native forms u and v with exogenous retinol to a greater extent than native form z can be converted to native form y. Because native forms u, v, w, and x appear to

be native forms of isoelectric variant 1 from denaturing 2D-PAGE and native forms y and z appear to be native forms of isoelectric variant 2 from denaturing 2D-PAGE, these results suggest that although both isoelectric variants 1 and 2 from denaturing 2D-PAGE appear to be capable of binding exogenous retinol, they may differ in their affinity for retinol. Retinol binding by isoelectric variants of RBP would be influenced by both binding capacity (i.e., number of binding sites) and the affinity of each isoelectric variant for retinol. X-ray crystallography indicates that RBP has a single binding site for retinol (27), so binding capacity greater than one retinol per RBP molecule is unlikely. Binding capacity less than one retinol molecule per molecule of RBP is possible and would indicate that some of the RBP molecules no longer bind retinol. In other words, some fraction of the protein has an affinity for retinol of zero, whereas the rest retains its affinity for retinol. The methods used here cannot distinguish between a uniform difference in affinity between the different isoelectric variants and a difference in the content of a fraction of each isoelectric variant that no longer binds retinol (i.e., a combination of normal affinity and zero affinity). Scatchard or other kinetic analysis will be required to distinguish between these possibilities. Nevertheless, in either case, the net result would be a difference in retinol binding for the different isoelectric variants. This difference could influence retinol delivery to tissues, given that these relationships are maintained *in vivo*. As with any experiment in which proteins are separated or otherwise processed, the results obtained could be artifacts of the procedures used. However, the procedures used for the purification and examination of protein forms in this experiment required no harsh treatment of the protein, decreasing the likelihood that the results are artifactual. The mechanism by which the isoelectric variants are generated is not known. Stallings-Mann et al. (16) have presented evidence that only one mRNA for RBP is found in pig endometrium. Therefore, the isoelectric variants of RBP are probably the result of posttranslational modification. It has been suggested that spontaneous deamidation may be the mechanism of generation of the isoelectric variants of RBP (16,26), but a systematic analysis is lacking. Given that the differences between the isoelectric variants observed occur *in vivo*, the results presented above suggest that the conversion of isoelectric variant 1 to isoelectric variant 2 could increase free retinol at the site of conversion. These results suggest that the mechanism of conversion of RBP into the various isoelectric variants warrants further investigation.

The finding that the isoelectric variants may differ in their affinity for retinol contrasts a previous report (15). Previous authors used ion-exchange HPLC to examine retinol-free vs. retinol-bound forms of RBP in relatively impure samples of RBP from uterine flushings. No attempt was made to correlate the protein peaks obtained by HPLC with isoelectric variants observed by 2D-PAGE; it is, therefore, impossible to determine how the saturation of each isoelectric variant changed after the addition of retinol. Furthermore, these results indicate that it may be difficult to separate the isoelectric variants by native charge alone because the native retinol-free forms of isoelectric variant 1 and the native retinol-bound form of isoelectric variant 2 have very similar native isoelectric points. Thus, the previous experiment is inconclusive.

In conclusion, RBP has been purified from porcine allantoic fluid and specific antiserum to this protein has been generated. Allantoic fluid and serum RBP have been compared, and unlike serum RBP, allantoic fluid RBP consists of multiple isoelectric variants that may differ either in their overall affinity or through the existence of a different proportion of each isoelectric variant having zero affinity for retinol. It is, therefore, possible that the formation of the isoelectric variants may play a role in retinol delivery

to tissues. Purified RBP should be useful for studies of the function of this protein during pregnancy.

ACKNOWLEDGMENTS/FOOTNOTES

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

REFERENCES

1. Roberts AB, Sporn MB. Cellular biology and biochemistry of the retinoids. In: *The Retinoids*, Vol. 2, Roberts AB, Sporn MB (eds). Academic Press, San Diego, p. 209–286, 1984.
2. DeLuca LM. Retinoids and their receptors in differentiation, embryogenesis and neoplasia. *FASEB J* 5:2924–2933, 1991.
3. Dingle JT, Fell HB, Goodman DS. The effect of retinol and of retinol-binding protein on embryonic skeletal tissue in organ culture. *J Cell Sci* 11:393–402, 1972.
4. Futterman S, Heller J. The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β -lactoglobulin, and the retinol-binding protein of human plasma. *J Biol Chem* 247:5168–5172, 1972.
5. Smith JE, Goodman DS. Retinol-binding protein and the regulation of vitamin A transport. *Fed Proc* 38:2504–2509, 1979.
6. Pervaiz S, Brew K. Homology of β -lactoglobulin, serum retinol-binding protein and protein HC. *Science* 228:335–337, 1985.
7. Papiz MZ, Sawyer L, Elropoulos EE, North ACT, Findlay JBC, Sivaprasadarao R, Jones TA, Newcomer ME, Kraulis PJ. The structure of β -lactoglobulin and its similarity to plasma retinol binding protein. *Nature* 324:383–385, 1986.
8. Schubert D, LaCorbiere M, Esch F. A chick neural retina adhesion and survival molecule is a retinol-binding protein. *J Cell Biol* 102:2295–2301, 1986.
9. Pervaiz S, Brew K. Homology and structure-function correlations between α_1 -acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J* 1:209–214, 1987.
10. Goodman DS. Vitamin A and retinoids: Recent advances, introduction, background and general overview. *Fed Proc* 38:2501–2503, 1979.
11. Soprano DS, Soprano KJ, Goodman DS. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J Lipid Res* 27:166–171, 1986a.
12. Soprano DS, Soprano KJ, Goodman DS. Retinol-binding protein and transthyretin mRNA levels in visceral yolk sac and liver during fetal development in the rat. *Proc Natl Acad Sci, USA* 83:7330–7334, 1986b.
13. Makeover A, Soprano DR, Wyatt ML, Goodman DS. Localization of retinol-binding protein messenger RNA in the rat kidney and in perinephric fat tissue. *J Lipid Res* 30:171–180, 1989.
14. Adams KL, Bazer FW, Roberts RM. Progesterone-induced secretion of a retinol-binding protein in the pig uterus. *J Reprod Fertil* 62:39–47, 1981.
15. Clawitter J, Trout WE, Burke MG, Araghi S, Roberts SM. A novel family of progesterone-induced, retinol-binding proteins from uterine secretions of the pig. *J Biol Chem* 265:3248–3255, 1990.
16. Stallings-Mann ML, Trout WE, Roberts RM. Porcine uterine retinol-binding proteins are identical gene products to the serum retinol binding protein. *Biol Reprod* 48:998–1005, 1993.
17. Harney JP, Mirando MA, Smith LC, Bazer FW. Retinol-binding protein: A major secretory product of the pig conceptus. *Biol Reprod* 42:523–532, 1990.
18. Trout WE, McDonnell JJ, Kramer KK, Baumbach GA, Roberts RM. The retinol-binding protein of the expanding pig blastocyst: Molecular cloning and expression in trophectoderm and embryonic disc. *Mol Endocrinol* 5:1553–1540, 1991.
19. Vallet JL, Christenson RK, Bartol FF, Wiley AA. Effect of treatment with retinyl palmitate, progesterone, oestradiol and tamoxifen on secretion of a protein similar to retinol-binding protein during uterine gland development in neonatal pigs. *J Reprod Fertil* 103:189–197.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275, 1951.
21. Selvaraj RJ, Sushella TP. Estimation of serum vitamin A by a microfluorometric procedure. *Clin Chim Acta* 27, 165–170, 1970.
22. Buhi WL, Vallet JL, Bazer FW. Denovo synthesis and release of polypeptides from cyclic and early pregnant porcine oviductal tissue in explant culture. *J Exp Zool* 252:79–88, 1989.

23. Roberts RM, Baumbach GA, Buhi WC, Denny JB, Fitzgerald LA, Babelyn SF, Horst MN. Analysis of membrane polypeptides by two-dimensional polyacrylamide gel electrophoresis. In: *Molecular and Chemical Characterization of Membrane Receptors*, Vol. 3, Venter JC, Harrison LC (eds). Alan R. Liss, Inc., New York, p. 61–113, 1984.
24. Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979.
25. Roberts RM, Raub TJ, Bazer FW. Role of uteroferrin in transplacental iron transport in the pig. *Fed Proc* 45:2513–2518, 1986.
26. Raz A, Shiratori T, Goodman DS. Studies on the protein-protein and protein-ligand interactions involved in retinol transport in plasma. *J Biol Chem* 245:1903–1912, 1970.
27. Newcomer ME, Jones TA, Aquist J, Sundelin J, Eriksson U, Rask L, Peterson PA. The three-dimensional structure of retinol-binding protein. *EMBO J* 3:1451–1454, 1984.
28. Vallet JL. Technical note: A radioimmunoassay for porcine retinol binding protein. *J Anim Sci* 72:2449–2454, 1994.