MULTIPLE RETINOL BINDING PROTEINS IN RABBIT LUNG

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SUMMARY

Rabbit lungs contain components sedimenting on sucrose gradients with a sedimentation coefficient of 2S that bind retinol with high specificity. DEAE-cellulose chromatography following gel filtration reveals the presence of 3 binding components of 17,000, 15,000 and 14,000 daltons respectively, showing different fluorescence spectra. One of the forms (B), here purified 700 fold, binds retinol specifically, as [^{3}H] - retinol can be displaced by non-radioactive retinol but not by retinal or retinoic acid. The fluorescence spectrum of a heptane extract of component B indicates it carries retinol as a ligand when isolated from the tissue.

Vitamin A (retinol) has been shown to influence normal differentiation of the epithelial tissue (1). Deficiency of this vitamin induces metaplasia in the tracheal epithelium (2) as well as pronounced changes in electrophoretic pattern of RNA molecules synthesized in vitro by tracheal explants (3). These and other results (4,5) suggest that vitamin A influences the transcriptional mechanism. Recently a report from this laboratory presented evidence of a retinol binding component present in the soluble part of cells from different rat tissues (6). Protein nature, affinity to retinol and specifity of this binding component make this protein a candidate for an intracellular receptor as described for steroid hormones (7).

Here we report detection, partial purification and characteristics of multiple retinol binding proteins from rabbit lungs. Rabbit was chosen as the experimental animal because it has been used as a model

system for the developing lung especially in connection with the appearance of newborn respiratory distress syndrome (8). This syndrome is characterized by a pathologically elevated surface tension in the lungs due to absence of surfactant material. Vitamin A has been reported to have influence on surface activity of the lungs (9). The mechanism and reason for the appearance of this phenomenon is obscure.

MATERIALS AND METHODS

All-trans-1-[^{3}H]-retinol with specific activity of 1.25 Ci/mmole was purchased from New England Nuclear. Purity was assayed by thin layer chromatography before use (10).

Retinol, retinal, and retinoic acid were obtained from Sigma Chemical Co. Appropriate solutions were prepared in ethanol containing 1 mg α-tocopherol per ml. All other chemicals were reagent grade.

Sephadex G-75 was from Pharmacia and DEAE-cellulose (DE-52) was obtained from Whatman.

Rabbit lungs were obtained from Pel-Freez Biologicals. The lungs after thawing were passed through a meat grinder, then homogenized for 30 sec. in a Waring blender with 2 volumes (v/w) of 0.05M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 10,000xg in a refrigerated centrifuge, then at 100,000xg to obtain the cytosol preparation. The cytosol was lyophilized to dryness and stored at -40°C before use.

Radioactivity was measured using a Beckman Model LS-233 scintillation counter with 5 ml of cocktail composed of toluene, Triton X-100 and Spectrafluor (237:123:10) mixed with 0.7 ml of sample. Efficiency of counting was 25%.

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer.

Sucrose gradient centrifugation was performed as described earlier (6).

The solutions were concentrated using an Amicon ultrafiltration device equipped with a UM-10 membrane.

RESULTS AND DISCUSSION

When rabbit lung cytosol is incubated at 4°C for 4 hours with all-trans-1-[^{3}H]-retinol (80nM), then layered on a 5-20% sucrose gradient, a binding component can be detected which shows sedimentation behavior (2S) corresponding to that observed for a retinol binding component present in various rat tissues (6).

The binding component was then partially purified by gel chromatography on Sepnadex G-75. To label the binding component the samples were incubated with 1-[^{3}H]-retinol for 30 min immediately prior to application to the column (Fig. 1). After Amicon ultrafiltration of fractions 63-72, the concentrated protein solution was chromatographed on a DEAE-cellulose column (Fig. 2, top), revealing two binding components, designated A and B.

If the samples were not labeled with [^{3}H]-retinol, the retinol binding fractions could be collected by elution position from the now standardized G-75 column. When such fractions which had not now been exposed to any exogenous retinol were submitted to DEAE-cellulose chromatography, three components with retinol-type fluorescence were observed. Two corresponded to the [^{3}H] labeled components A and B, the third component (C) eluting later (Fig. 2, bottom).

The fluorescence excitation spectra of the three partially purified

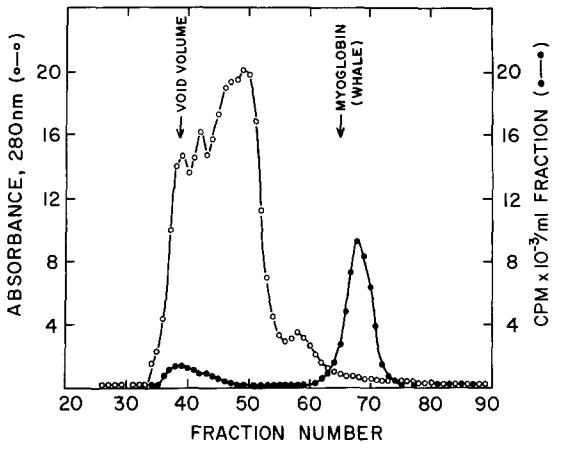


Fig. 1 Separation of retinol binding protein by gel filtration on a Sephadex G-75 column (5.0 x 76 cm). The sample (2.7 gm cytoplasmic protein dissolved in 50 ml 0.05 M Tris-HCl buffer pH 7.6, 0.2 M NaCl) was incubated with 1.2 n moles (1.5 μCi) [^{3}H]-retinol for 30 min before application to the column. The column was eluted with the same buffer. Fractions of 15 ml were collected and assayed for protein by absorbance at 280 nm and retinol binding by scintillation counting.

components are shown in Fig. 3. As can be seen each component has a somewhat different excitation spectrum, but all are similar to that observed for retinol bound to serum retinol binding protein of human (11).

Furthermore, when the molecular weights of these separated components were determined by gel filtration as shown in Fig. 4, apparent molecular sizes of 17,000, 15,000 and 14,000 daltons were found for peak A, B, and C, respectively. This suggests they also differ by size. This size range is similar to that of 16,000 daltons determined for the tissue binding component in the rat (6).

Retinol binding protein have been detected in serum (12). As juaged by gel filtration the molecular sizes of serum retinol binding proteins from various species are significantly larger than the tissue binders described here. Reported values are 20,000 daltons for rat

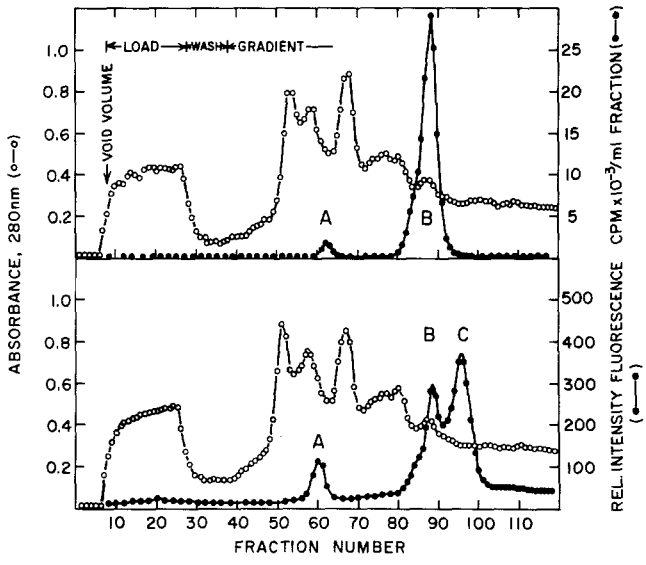
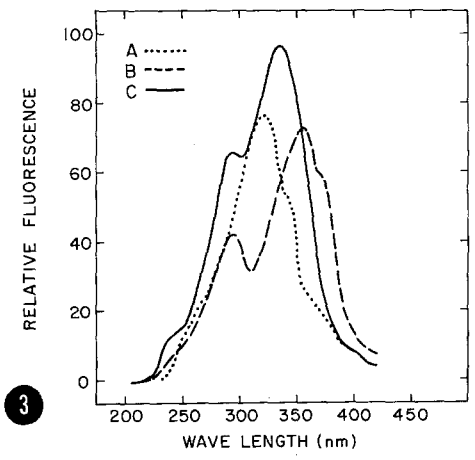


Fig. 2 Purification of retinol binding protein by chromatography on a DEAE-cellulose column (1.6X 40cm) of the fractions collected after Sephadex G-75 chromatography. The samples (105 ml containing 270 mg protein labeled with [^{3}H]retinol prior to chromatography on Sephadex G-75, top; 100 ml containing 280 mg protein not exposed to any exogenous [^{3}H] retinol, bottom) were exhaustively dialyzed against 0.01 M Tris-HCl, pH 7.7 and applied to the columns equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl from 0 to 0.2 M (total volume, 500 ml). Fractions of 5.5 ml were collected at a flow rate of 53 ml per hour. Protein was assayed by absorbance at 280 nm. Retinol binding was determined by liquid scintillation counting of radioactivity (top) or by fluorescence at 460 nm with excitation at 330 nm (bottom).

(12), 21,000 daltons for human (12) and 20,000 daltons for dog (13).

Special attention has been given to component B. The extent and progress of its purification is shown in Table 1. It represents less than 0.002% of the soluble protein of rabbit lung.

When fraction B, not exposed to exogenous retinol at anytime in its purification, was extracted for 2 hours by stirring rapidly with an equal volume of heptane under N_2, the heptane extract exhibited a fluorescence excitation spectrum identical to that of authentic all-trans



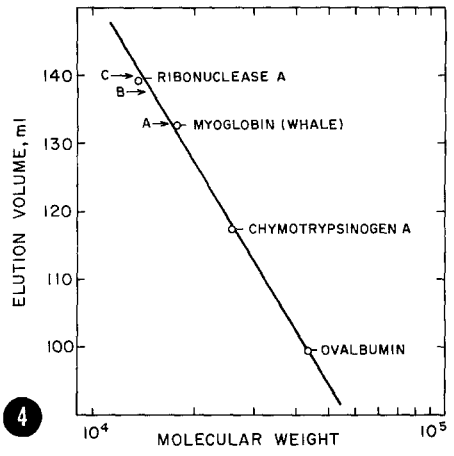


Fig. 3 Fluorescence excitation spectra of component A, fraction 60; component B, fraction 87; and component C, fraction 97. Emission was measured at 460 nm. Fraction numbers relate to those in Fig. 2 (bottom).

Fig. 4 Determination of molecular weights of components A, B, and C by chromatography on a column of Sephadex G-75 (2.5 x 41 cm) calibrated with the indicated standard proteins. Elution volume of components A, B, and C, indicated by the arrows was determined by measurement of fluorescence at 460 nm, with excitation at 330 nm. The load volume was 2.0 ml. Fractions of 3.0 ml were collected. Elution buffer described in legend to fig. 1.

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retinol (Fig. 5) indicating that the binding component carries all-trans retinol as a ligand in the cell.

The partially purified component B has been incubated with all-trans-1-$[^3H]$-retinol, as indicated in Fig. 6, in the presence and absence of a 200 fold greater concentration of cold retinol, retinal, and retinoic acid. The sucrose gradient profiles show clearly that only retinol is able to compete with the binding of $[^3H]$-retinol indicating the specificity of the interaction of the binding component with retinol, as observed earlier with preparations from rat tissue.

When component C is incubated with $[^3H]$-retinol and subjected

TABLE I

Partial Purification of Lung Retinol Binding Protein

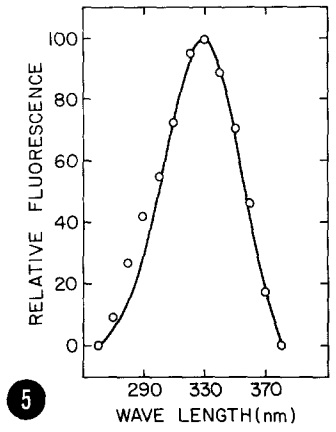
Fraction	Protein (tot. mg)	Retinol Binding Protein* (tot. μg)	Sp. Activity# pmole bound /mg protein	Yield
Cytosol	14,120	233	1.1	
Sephadex Peak	262	179	45.6	79
DEAE Peak B	10.4	109	700	47.5

* Based on a molecular weight of 15,000 daltons and one binding site per molecule.

\# $[^3H]$ -Retinol binding based on total radioactivity in the 2S peak observed after sucrose gradient centrifugation less the radioactivity present in the presence of a 200-fold excess of unlabeled retinol.

to sucrose gradient centrifugation, binding in the 2S region is observed, although C was not labeled when the cytosol was exposed to $[^3H]$ -retinol prior to purification. When a equivalent amount of component B, based on intensity of fluorescence, is assayed for binding in the same system, much more $[^3H]$ -retinol is bound in the 2S peak. This may indicate that the retinol bound by component C exchanges less readily with added $[^3H]$ -retinol compared to component B.

The function of these three components is not known. It can not be excluded that the multiple forms detected here could originate by partial degradation of a single component.



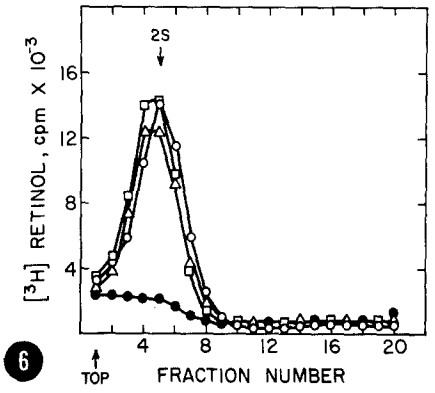


Fig. 5 Fluorescence excitation spectrum of authentic all-trans retinol in heptane (——) and of the heptane extract of component B, tube 88 (0). Fraction number extracted relates to fig. 2 (bottom). Emission was measured at 480 nm.

Fig. 6 Sucrose gradient centrifugation of 10 μg protein from peak B, incubated with 80 nM [^{3}H] retinol in the presence or absence of a 200 fold excess of cold retinol, retinal, or retinoic acid. The gradients were centrifuged for 20 hrs. at 189,000xg. Myoglobin (2S) on a separate gradient was used as a marker. Control (0); + retinol (●); + retinal (□); + retinoic acid (Δ).

The binding components are quite stable; the tissue or preparations from the purification procedure can be stored frozen with no apparent loss of binding ability. Though the amount of binding component recovered from the lung tissue is small, the stability suggests further purification. Studies with the purified components may shed light on their possible participation in the mechanism of action of Vitamin A. Differences between the tissue binding components and that shown to be present in the serum are being investigated.

ACKNOWLEDGEMENT

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