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In Vitro Binding of Retinol to Rat-Tissue Components

(density gradient centrifugation/gel filtration/retinal/retinoic acid/serum)

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ABSTRACT **The high-speed supernatant fraction of rat liver, lung, kidney, testis, and intestinal mucosa contains a component capable of binding [^{3}H]retinol *in vitro* when binding is analyzed by sucrose density gradient centrifugation or gel filtration. This binding component can be distinguished from one identified in rat serum. Whereas the tissue component sediments in the 2S region of sucrose gradients, the serum component sediments in the 4.6S region. Molecular weight estimations by gel filtration indicate molecular weights of 16,000 and 67,000 for the tissue and serum binding components, respectively. Unlabeled retinol, but not retinoic acid, competes for the binding of [^{3}H]retinol in tissue cytosols. Competition for the binding of [^{3}H]retinol by unlabeled retinal has also been observed in tissue cytosols, but may result from the *in vitro* reduction of retinal to retinol. Unlabeled retinol, retinal, and retinoic acid fail to compete for the binding of [^{3}H]retinol in serum under the conditions used. The tissue binding component (testis) is sensitive to digestion with Pronase, but not with RNase or DNase, indicating a protein nature for this component.**

The only well-defined function of vitamin A in vertebrates is its involvement in vision (1) in the form of retinol and retinal. Vitamin A is also needed for normal skeletal development and reproduction (2). That the vitamin is necessary for normal differentiation of epithelial tissues is also well established (3–5), although the mechanism(s) by which cytodifferentiation is regulated remain obscure. There is, however, increasing evidence that protein synthesis and RNA metabolism are affected in deficient animals. Thus, administration of vitamin A to animals deficient in this compound results in increased incorporation of radioactive precursors into total cellular and nuclear RNA of several tissues (6–9). RNA preparations from liver nuclei of normal rats and rats deficient in vitamin A differ (10) as do RNA preparations synthesized *in vitro* by hamster tracheal epithelium (11).

Results comparable to these, at least qualitatively, have been found after administration of other physiologically important compounds. An early effect of the administration of many steroid hormones is the alteration of RNA synthesis in the nuclei of target tissue cells (12–14). These results suggest that many of the biological effects of steroid hormones may be mediated by control of nuclear metabolism, in particular, the regulation of gene transcription.

Although the actual mechanism(s) by which steroids regulate gene activity remains obscure, certain aspects of the hormone–tissue interaction have been elucidated. Once the steroid enters the cell it binds to specific "receptor" molecules initially present in the cytosol (15, 16). Such "receptor" proteins have been identified for several steroids (15–24). The hormone–"receptor" complex may then enter the cell nucleus and interact with nuclear components (25–28), effecting, in some way, an alteration of gene transcription.

We were prompted by the above considerations to examine tissues of rats for the presence of a macromolecule that might be involved in mediating the tissue's response to vitamin A by first binding the vitamin. During our investigations a rat-serum transport protein, retinol-binding protein, was found and partially characterized (29, 30).

We report here the finding of a macromolecular fraction present in lung, liver, kidney, testis, and intestinal mucosa of rats which is capable of binding [^{3}H]retinol *in vitro* and which differs from the serum component that binds [^{3}H]-retinol *in vitro*.

METHODS AND MATERIALS

Serum and Cytosol Preparation. Male Holtzman rats, fed freely on Lab Chow (Ralston Purina Co.), were used in all experiments. Blood was collected by cardiac puncture of animals anesthetized by ether and allowed to clot at 0° for 15–20 min. The liver and intestine were removed from animals starved for 12–14 hr. Livers were perfused with cold 0.9% NaCl before homogenization. Intestinal mucosal cells were collected after the intestine was rinsed with cold 0.9% NaCl. The intestine was placed on a chilled glass plate and slit open; the mucosa was scraped off with a spatula. All tissues removed from the animal were homogenized in 50 mM Tris·HCl (pH 7.5), 4 ml per g of tissue, with a glass–Teflon tissue homogenizer.

Clotted blood and tissue homogenates were centrifuged at 31,000 × *g* for 10 min in a refrigerated centrifuge. The supernatant factions were collected and centrifuged at 105,000 × *g* for 60 min at 4° to yield high-speed supernatant (cytosol) or serum preparations. In some experiments the serum was diluted with 50 mM Tris·HCl (pH 7.5) before use in incubation mixtures.

Cytosol Incubations. [^{3}H]Retinol (1.25 Ci/mmol, New England Nuclear Corp.) dissolved in ethanol was added in 5 μl to 1.0 ml of cytosol (40 nmol, about 26,000 cpm/ml of cytosol) or serum, mixed thoroughly, covered with Parafilm, and incubated in the dark at 0–4°. Although a 4-hr incubation was sufficient to obtain maximum binding in liver cytosol preparations, all samples were incubated for 12–16 hr. In the binding competition experiments, the unlabeled compound was added to the incubation mixture dissolved in 5 μl of ethanol; solvent alone was added to control mixtures.

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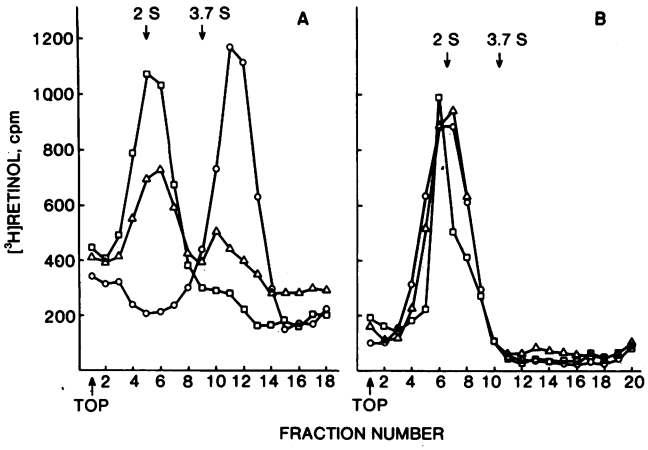

FIG. 1. Sucrose gradient centrifugation of serum (diluted 1:6) and liver and testis cytosols (*A*) and kidney, lung, and intestinal mucosal cytosols (*B*) after incubation with 40 nM [³H]retinol. Myoglobin (2 S) and ovalbumin (3.7 S) on separate gradients were used as markers. Centrifugation was for 18 hr at 189,000 × *g*. (*A*) Serum (○); liver cytosol (△); testis cytosol (□). (*B*) Kidney cytosol (○); lung cytosol (△); intestinal mucosal cytosol (□).

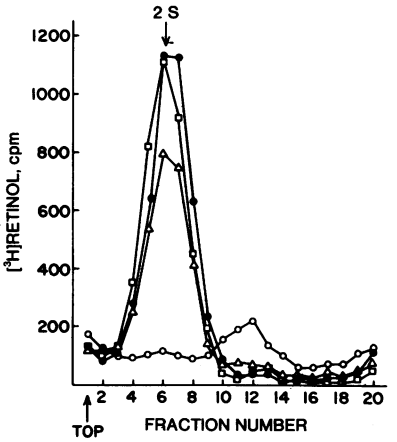

FIG. 3. Sucrose gradient centrifugation of testis cytosol after incubation with 40 nM [³H]retinol in the presence or absence of a 200-fold excess of unlabeled retinol, retinal, or retinoic acid. Samples were incubated as in *Methods;* centrifugation was for 18 hr at 189,000 × *g*. Control (●); +200-fold excess of retinol (○); + 200-fold excess of retinal (△); + 200-fold excess of retinoic acid (□).

Sucrose Gradient Centrifugation. Binding of [³H]retinol to components of tissue cytosol or serum was analyzed by sucrose density gradient centrifugation on linear 5–20% (w/v) sucrose gradients in 10 mM Tris·HCl–1 mM EDTA–10 mM thioglycerol–10 mM KCl (pH 7.5). After the cytosols or serum were incubated with [³H]retinol as described above, 0.2-ml aliquots of the incubation mixture were applied to gradients at 4° and centrifuged for 18–19 hr at 158,000 × *g* or 189,000 × *g* in a Spinco SW 50.1 rotor at 4°. Whale skeletal-muscle myoglobin (Sigma Type II) and crystalline ovalbumin (Sigma Grade V), molecular weight 17,830, 1.98 S (31) and molecular weight 45,000, 3.66 S (32), respectively, were applied to separate gradients as standards for determination of sedimentation constants (33). After centrifugation, the gradients were fractionated by piercing the bottom of the tube and collecting 0.25-ml fractions in counting vials. To each vial was added 0.5 ml of water and 5 ml of a liquid scintillation cocktail containing toluene–Triton X-100–Spectrofluor (Amersham/Searle) in the ratio 2362:1230:100 (v/v/v). The counting efficiency was about 30% for ³H. The positions of the standard proteins on the gradients were determined by monitoring UV absorbance of each fraction.

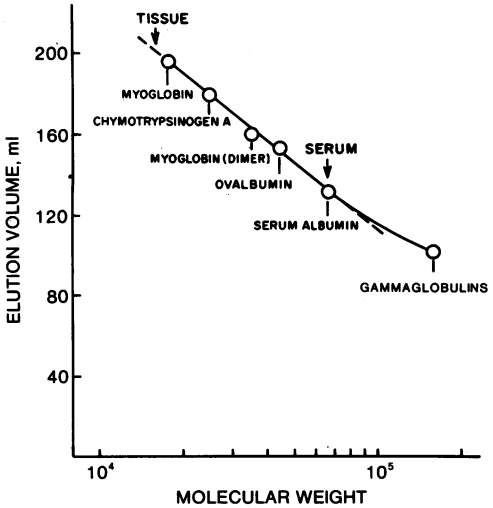

FIG. 2. Elution volumes of standard proteins from a 2.5 × 50-cm column of Sephadex G-100 as a function of $\log_{10}$ molecular weight. The elution volumes of the tissue (lung, liver, testis) component binding [³H]retinol and the serum-binding component are indicated by *arrows*.

Molecular-Weight Estimation on Sephadex G-100. The molecular weight of the [³H]retinol-binding components of serum and liver, testis, and lung cytosols were estimated by gel filtration on Sephadex G-100 by the method of Andrews (34). A 2.5 × 50 cm column of Sephadex G-100 was calibrated by determining the elution volume of sperm-whale myoglobin (molecular weight 17,800), chymotrypsinogen A (molecular weight 25,000), ovalbumin (molecular weight 45,000), bovine-serum albumin (molecular weight 67,000), and human gamma globulins (average molecular weight 160,000) (Schwarz/Mann). The total column volume and the void volume were determined by elution of [³H]retinol (molecular weight 286) and blue dextran 2000 (average molecular weight 2,000,000). Serum and tissue cytosols prepared as described above were diluted with an equal volume of 50 mM Tris·HCl–0.2 M KCl (pH 7.5) and incubated for 12–16 hr with [³H]retinol (40 nM) at 4° before gel filtration at room temperature. 3-ml Fractions were collected, and the protein content of each was estimated by measuring the absorbance at 280 nm. Elution of [³H]retinol was monitored by liquid scintillation spectrometry of 0.5-ml aliquots from each fraction in 5 ml of scintillation cocktail.

Binding Assay. For routine determination of bound [³H]-retinol in serum or tissue cytosols, an aliquot (0.1 ml) of the incubation mixture was applied to a column (1.1 × 5.5 cm) of Sephadex G-25 Fine equilibrated with 50 mM Tris·HCl (pH 7.5). The sample was eluted with the same buffer at a

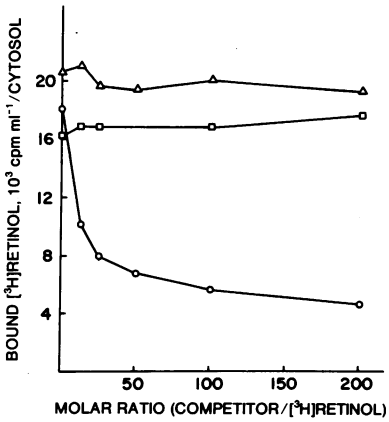

FIG. 4. Competition for the binding of [³H]retinol (40 nM) in testis cytosol by increasing concentrations of retinol, retinal, and retinoic acid. Samples were incubated and the binding of [³H]-retinol was analyzed as in *Methods*. Unlabeled competitors were retinol (○), retinal (△), and retinoic acid (□). Different cytosol preparations were used for each competitor.

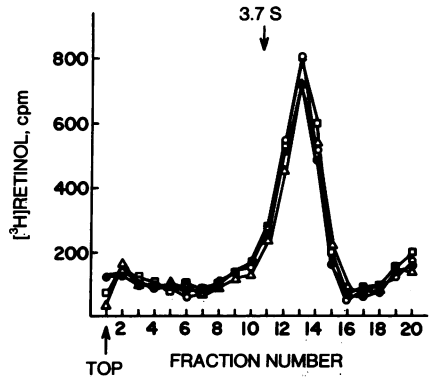

FIG. 5. Sucrose gradient centrifugation of serum (diluted 1:10) after incubation with 40 nM [³H]retinol in the presence or absence of a 200-fold excess of unlabeled retinol, retinal, or retinoic acid. The gradients were centrifuged for 18 hr at 158,000 × *g*. Control (●); + retinol (○); + retinal (△); + retinoic acid (□).

flow rate of about 25 ml/hr; 0.5-ml fractions were collected. The fractions were then transferred to counting vials, mixed with 5 ml of the liquid scintillation cocktail, and counted. [³H]Retinol eluted from the columns in the void volume was considered "bound" retinol and was clearly separated from the "free" [³H]retinol appearing in later fractions.

Enzyme Digestion Studies. The stability of the binding of [³H]retinol to the testis-binding component was examined by sucrose density gradient centrifugation after incubation with Pronase (nuclease-free; Calbiochem), bovine pancreatic RNase (RNase A), and bovine pancreatic DNase (DNase I; Worthington). [³H]Retinol was added to 1.0-ml aliquots of testis cytosol to give a final concentration of 40 nM and incubated for 16 hr at 4°. Pronase or RNase (200 μg) was added to the incubation mixtures in 0.1 ml of 50 mM Tris·HCl (pH 7.5); an equal volume of buffer only was added to the control tubes. DNase I (200 μg) plus $MgCl_2$ (5.5 μmol) was added to another incubation mixture in 0.1 ml of 50 mM Tris·HCl (pH 7.5); the control contained $MgCl_2$ in the buffer. The tubes were then incubated at 25° for 3 hr before sucrose gradient centrifugation.

RESULTS

Detection of the Binding Component. Sucrose density gradient analysis of the binding of [³H]retinol to components of tissue cytosols and serum (1:6 dilution) is presented in Fig. 1*A* and *B*. The data indicate the binding of labeled retinol to a macromolecular fraction present in liver, testis, kidney, lung, and intestinal mucosal cytosols which sediments in the 2S region of the gradients with myoglobin (2 S) as a standard. This result is in contrast to the result with serum, where the labeled retinol is associated with a component(s) sedimenting in the 4.6–4.8S region with ovalbumin (3.7 S) as a standard. Occasionally a slight peak of [³H]retinol with sedimentation properties of the serum binding component has been seen on gradients of tissue cytosols although in no instance has a 2S binding component been observed in serum preparations.

Molecular Weight of the Binding Component. An approximate molecular weight may be calculated from the sedimentation data for the tissue binding component and the serum binding component with myoglobin and ovalbumin as the respective standards (33). With 2 S as the sedimentation coefficient for the tissue-binding components and 4.6 S as the sedimentation coefficient of the serum binding component, molecular weights of 18,000 and 63,000 are obtained for the tissue and serum components, respectively.

The molecular weights of the binding components of serum and liver, testis, and lung cytosols were also estimated by Sephadex gel filtration. The tissue binding components were eluted from the column in an elution volume corresponding to a protein with a molecular weight of 16,000, while the serum binding component showed a molecular weight of 67,000 (Fig. 2). These values are in good agreement with the molecular weights calculated from the sedimentation coefficients.

Ligand Specificity of the Binding Component. The ability of unlabeled compounds to compete for the binding of [³H]-retinol was examined by sucrose density gradient analysis and by the binding assay by use of Sephadex gel filtration. When testis cytosol was incubated with 40 nM [³H]retinol in the presence of a 200-fold excess of unlabeled retinol, the 2S peak of bound [³H]retinol was virtually eliminated (Fig. 3)

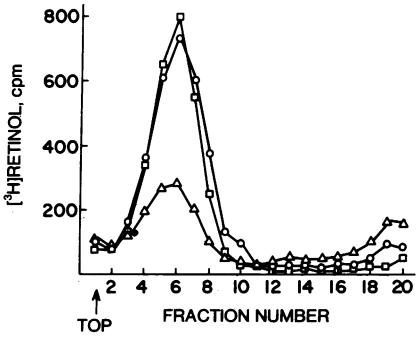

FIG. 6. Sucrose gradient centrifugation of testis cytosol after incubation with 40 nM [³H]retinol for 18 hr at 4° and subsequent incubation for 3 hr at 25° in the presence or absence of Pronase or RNase. Gradients were centrifuged for 19 hr at 158,000 × *g*. Control (○); + Pronase (△); + RNase (□).

and a slight peak of [^{3}H]retinol with sedimentation properties of the serum-binding component appeared. The presence of a 200-fold excess of retinoic acid did not alter the binding of [^{3}H]retinol to the 2S component, while the presence of a 200-fold excess of retinal did reduce the binding somewhat, relative to the control sample. As is evident in Fig. 3, recovery of free [^{3}H]retinol from the gradient tubes was low (probably due to adherence to the Polyallomer tubes used for centrifugation).

The binding of [^{3}H]retinol in tissue cytosols in the presence of increasing amounts of unlabeled retinol, retinal, and retinoic acid was also measured by the gel filtration assay. The data presented in Fig. 4 for testis cytosol indicate that increasing concentrations of retinol up to a certain excess progressively reduced the binding of [^{3}H]retinol. Increasing concentrations of retinal over the range tested (0- to 200-fold excess) had little if any effect, while retinoic acid had no effect on the binding of [^{3}H]retinol. The apparent discrepancy between the results of competition studies with retinal as the competing ligand (Figs. 3 and 4) most likely results from the inherent differences of the two assays for binding. Sucrose gradient centrifugation measures [^{3}H]retinol bound to specific components (Fig. 3), while the gel filtration assay measures total bound [^{3}H]retinol. Thus, in the latter case, small amounts of [^{3}H]retinol displaced from the 2S binding component could be bound by other macromolecules (as is seen in Fig. 3 with retinol as competitor) and thereby reduce or mask any competition actually occurring at the binding sites of the 2S component.

When perfused liver cytosol was used in experiments identical to those illustrated in Fig. 4, retinal competed almost as effectively as retinol, while retinoic acid again did not compete. Preliminary experiments indicate that at least part of the competition for [^{3}H]retinol binding in liver cytosol by retinal resulted from the *in vitro* reduction of retinal to retinol.

The ability of excess unlabeled retinol, retinal, and retinoic acid to compete for the serum binding sites for [^{3}H]retinol was also examined. The sucrose gradient centrifugation data presented in Fig. 5 indicate that the binding of [^{3}H]retinol in the 4.6S region of the gradient is completely unaffected by the presence of a 200-fold excess of unlabeled retinol, retinal, or retinoic acid. These results were obtained with a 10-fold dilution of serum in the incubation mixture. Undiluted serum or serum diluted 1:5 gave similar patterns.

Protein Character of the Binding Component. Some insight into the nature of the tissue binding component was obtained from the study of the stability of the binding of [^{3}H]retinol after trreatment of the cytosol with Pronase, RNase, and DNase. The data presented in Fig. 6 indicate that the binding of [^{3}H]retinol in the 2S region was reduced by 57% after Pronase treatment, but was unaffected by RNase. Treatment of cytosol with DNase likewise had no effect on the binding of [^{3}H]retinol relative to the untreated control.

DISCUSSION

The nature of the binding of retinol to the cytosol and serum component is still under investigation. An accurate determination of the dissociation constants for the serum and cytosol binding components has not been attempted, but a reasonably high affinity for the ligand may be inferred from the persistence of the peaks of bound [^{3}H]retinol during the sucrose gradient centrifugation. A high affinity for the ligand is also an important characteristic of the steroid "receptor" proteins. On the other hand, the size of the tissue retinol-binding component is considerably smaller than that reported for the steroid "receptor" proteins. The latter have sedimentation constants of 4–8 S, depending upon the source (cytosol or nuclear fraction) and conditions of sucrose gradient centrifugation (14, 19, 35).

The binding of [^{3}H]retinol observed in tissue cytosols apparently occurs at sites specific for the retinol structure. In both testis and liver cytosols the binding of [^{3}H]retinol could be reduced considerably by including excess unlabeled retinol in the incubation mixtures. The addition of excess unlabeled retinal, but not retinoic acid, also reduced the binding of [^{3}H]-retinol in testis and liver cytosols. Experiments with a more highly purified system will be required to distinguish between the possibility that the tissue binding sites actually have an affinity for retinal that is lower than their affinity for retinol and the possibility that the competition results from the *in vitro* reduction of a limited amount of retinal to retinol.

Of the forms of vitamin A available to an animal, retinol is the most biologically active, followed by retinal, which is presumably converted to retinol in the intestine (36). Retinoic acid, however, apparently cannot be converted to retinol, but can nevertheless maintain the general well-being of the animal, with the exception of vision and reproductive capacity (37, 38). If the tissue binding component for retinol described here is essential for mediating the tissue response to vitamin A, then the biological activity of retinoic acid must be accounted for by the metabolism of this compound to a retinol-like structure which can be bound by the tissue component, or by the presence of another binding component for retinoic acid itself.

Unlike the binding of [^{3}H]retinol in tissue cytosols, the binding in serum was not competed for by a 200-fold molar excess of unlabeled retinol. The failure of retinol to compete may be indicative of a large number of binding sites in serum that are not saturated with the amounts of retinol used in these studies.

The enzyme digestion studies done with testis cytosol indicate that the 2S binding component for [^{3}H]retinol is most likely a protein molecule, since Pronase, but not RNase or DNase, reduced the binding of [^{3}H]retinol in testis cytosol.

Recently published studies of rat-serum retinol-binding protein (29, 30) indicate that it is similar to that described in human serum (39, 40). Both are about 20,000 molecular weight and are associated with prealbumin to form a complex of about 70,000 molecular weight. Retinol is the only form of vitamin A that can be detected in purified preparations of the serum complex. It has also been reported that rat liver contains material that is immunoreactive with antisera prepared against purified serum component (41, 42). The liver is believed to be the site of synthesis of the transport protein, while its secretion from this organ is apparently regulated by vitamin A levels in the animal (41, 42). It is of interest to determine any relationship that might exist between retinol-binding protein and the tissue binding components for retinol reported here. Preliminary evidence from our laboratory indicates that human lung cytosol also contains a component capable of binding [^{3}H]retinol *in vitro* that sediments in the same region of sucrose gradients as does the binding component from rat tissue. Likewise, sucrose gradient profiles of

the *in vitro* binding of [^{3}H]retinol in human and rat serum appear to be identical.

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