Factors Affecting the Binding of Retinol to Serum and Its Components[1] (34801)

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Retinol has been reported to circulate in plasma associated with a transport protein (1, 2, 4, 5). The purification and properties of a specific retinol-binding protein (RBP), have recently been described (3).

The present investigation was designed (1) to study the binding of retinol to human sera and to sera from normal, vitamin A-deficient, and vitamin A-supplemented rats, (2) to determine what factors affect this binding process, and (3) to investigate the binding of retinol to specific serum fractions.

Materials and Methods. Aqueous suspensions of the all-*trans* forms of ^{14}C-retinol (sp act 22 μCi per μmole), and ^{14}C-β-carotene (sp act 0.34 μCi per mmole) were prepared by taking appropriate aliquots from stock benzene solutions, evaporating under N_2, redissolving in approximately 2.0 ml of ethanol, and adding appropriate aliquots (ca. 40–50 μl) of the ethanolic solutions to 10 ml of 0.1 *M* sodium phosphate buffer, pH 7.7.

All sera were prepared from venous blood. Vitamin A-deficient rats were prepared as previously described (6). Elevated rat serum vitamin A levels were produced by daily, oral administration of 10,000–15,000 IU of water-miscible vitamin A for 3 days. Random human sera were obtained from the Clinical Laboratories, West Virginia University Hospital.

Radioassay was carried out by dissolving samples in Brays solution (7) and counting in a Packard Tri-Carb liquid scintillation spectrophotometer. Retinol content of serum samples was determined by the trifluoroacetic acid method (8). Serum protein was measured by the method of Lowry *et al.* (9).

The binding of ^{14}C-retinol to serum was measured by a Sephadex G-25, batchwise, semimicrotechnique, patterned after Pearlman and Crepy (10). Incubation mixtures consisted of 400 mg Sephadex G-25 (fine grade), 2 ml of 0.1 *M* sodium phosphate buffer, pH 7.7, 1 ml of ^{14}C-retinol solution (22 μCi/μmole), and 1 ml of diluted serum (1:10 to 1:60 with 0.1 *M* sodium phosphate buffer, pH 7.7). The incubations were carried out at 25° for 1 hr with agitation. A control tube was similarly prepared except that 1 ml of distilled water was added instead of serum. The amount of retinol bound, expressed as μmoles of retinol bound per mg protein, was determined by subtracting the retinol concentration of the supernatant fluid in the control tube from the retinol concentration of the supernatant fluid in the tubes containing serum. This binding technique was found to be reliable and valid.[3]

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[3] Proof that ^{14}C-retinol was bound to serum proteins was obtained by chromatography on columns of Sephadex G-25. Serum (0.5 ml) containing ^{14}C-retinol (40 μl) was chromatographed on Sephadex G-25 columns measuring 0.5 × 30 cm. Blue dextran was used to estimate the void volume (3.5 ml) and 1-ml fractions were eluted with 0.01 *M* sodium phosphate buffer, pH 7.7. In the absence of protein, the majority of the ^{14}C-retinol eluted in a volume of approximately 280 ml. In the presence of protein, 95% of the ^{14}C-retinol was associated with proteins traveling in the void volume. Moreover, retinol determination of the eluting fractions indicated that approximately 96% of the retinol was present in the protein fractions.

In order to study the contributions made by each serum protein fraction to the total serum binding, serum samples (0.5-ml) were incubated at 25° for 1 hr with 10 μl of ^{14}C-retinol and then fractionated by polyacrylamide-disc electrophoresis according to Davis (11). Individual serum protein fractions were horizontally sectioned from appropriate areas of each gel, eluted overnight with 0.1 *M* sodium phosphate buffer, pH 7.7, and counted in a liquid-scintillation spectrophotometer.

The amount of retinol bound to various serum protein fractions was determined by incubating each fraction with ^{14}C-retinol (see Table II), and determining the amount of retinol bound as described above.

The binding of serum to ^{3}H-retinoic acid (0.02 μCi per μmole) and ^{14}C-β-carotene (0.34 μCi per mmole) was determined by the same procedure as described for ^{14}C-retinol.

The effect of various unlabeled vitamin A derivatives on the serum binding of ^{14}C-retinol was studied by adding appropriate ethanolic solutions of the derivative to the incubation mixture (see Table III).

Results. The binding of retinol to various rat and human sera are shown in Table I. In comparison to normal rat serum, vitamin A-deficient serum was less effective in binding retinol, whereas serum from vitamin A supplemented rats was more effective. The quantities of retinol bound to human sera were similar to normal rat sera. Further analysis of these data indicates a direct relationship between the serum binding of retinol and the protein and/or retinol content of serum.

TABLE I. Effect of Protein Levels and Retinol Content of Serum on the Binding of ^{14}C-Retinol to Serum.

Diluted sera	Content per incubation: Protein (mg)	Content per incubation: Retinol[a] (μg)	μmole C^{14}-retinol bound $\times 10^2$
Normal rat (1:20)	2.1	0.019	2.5
Normal rat (1:60)	0.7	0.005	1.4
Normal rat (1:20)	2.5	0.026	2.8
Deficient rat (1:60)	0.7	0.001	1.7
Deficient rat (1:60)	0.7	0.002	1.6
Supplemented rat (1:20)	1.9	0.057	3.8
Supplemented rat (1:60)	0.6	0.020	2.6
Supplemented rat (1:60)	0.8	0.012	2.1
Human (1:50)	1.5	0.014	2.8
Human (1:50)	1.5	0.018	2.8
Human (1:50)	2.0	0.010	3.2

[a] The endogenous retinol content of the diluted serum.

TABLE II. The Binding of ^{14}C-Retinol to Human Serum Fractions.

Serum fraction[a]	μmole ^{14}C-retinol bound/mg protein $\times 10^{2b}$
γ-Globulin (fraction II)	3.4
β-Globulin (fraction III)	1.5
α-Globulin (fraction IV)	12.3
Albumin (crystallized)	6.0

[a] Commercially prepared serum proteins (Nutritional Biochemicals Co., Cleveland) were analyzed via disc electrophoresis and found to contain only the protein(s) of that given fraction.

[b] The amount of retinol bound was measured by the semimicro Sephadex G-25 technique as described in the Experimental section except that the human serum fractions (1%) were diluted (1:20 to 1:60) with 0.1 *M* sodium phosphate buffer, pH 7.7, and used in place of whole serum.

In order to determine the distribution of retinol among various serum proteins, normal rat and human sera, preincubated with ^{14}C-retinol, were subjected to polyacrylamide-gel electrophoresis. Approximately 21, 45, and 20% of the total recovered radioactivity (80%) were present in the γ-globulin, α-globulin, and albumin fractions, respectively. The β-globulin fraction contained less than 10% of the radioactivity.

In accord with the above findings, several commercial fractions of human serum were found to be capable of binding ^{14}C-retinol. Table II indicates that the α-globulin fraction bound more retinol than the albumin and γ-globulin fraction.

^{14}C-Retinol was not the only vitamin A derivative bound by serum. When analyzed via the Sephadex technique, human serum also bound ^{3}H-retinoic acid and ^{14}C-β-carotene in amounts of 2.4×10^{-3} and 0.6×10^{-3} μmoles per mg protein, respectively.

TABLE III. Serum Binding of ^{14}C-Retinol in the Presence of Unlabeled Vitamin A Derivatives.

Addition[a]	μmole ^{14}C-retinol bound/mg protein $\times 10^{3}$[b]	% Inhibition
None	7.3	0
All-*trans* retinal	4.9	33
13-*cis* retinal	7.3	0
All-*trans* retinoic acid	5.9	19
All-*trans* retinyl acetate	6.4	13

[a] Unlabeled vitamin A derivatives (0.01 μmole) dissolved in ethanol (10 μl) were added to the incubation mixtures, and the binding affinity of serum was determined as described in the text.

[b] Both human and rat sera were found to bind similar amounts of retinol per mg protein.

Several unlabeled vitamin A derivatives were found to inhibit the serum binding of retinol (Table III). The all-*trans* form of retinal was the most active inhibitor while retinoic acid and retinyl acetate were less effective in reducing retinol binding. The 13-*cis* form of retinal had no appreciable effect on the binding.

Discussion. Rat sera from normal, vitamin A-deficient, and vitamin A-supplemented animals bound varying amounts of retinol. The data indicate that these differences were related to both the protein and retinol content of serum. The reason for these relationships is not apparent. However, the effect of protein may be attributable in part to the levels of retinol-binding protein (RBP) in serum, which would influence the amount of retinol bound. On the other hand, the effect noted with various serum retinol concentrations is more difficult to explain. It may be that an increase in this ligand might serve to expose more available binding sites on the protein molecule. Similar studies (10) on the binding of testosterone to human serum disclosed that larger bindings occurred with pregnancy serum that with normal serum. These findings may be analogous to our results on the binding of retinol to serum from vitamin A-supplemented rats. Future studies on the interaction of vitamin A with RPB may serve to explain these effects.

It has been reported (2–5) that retinol is associated mainly with the α-globulin fraction of blood. Our results support this observation, however, we also find that the γ-globulin and albumin fractions can bind appreciable quantities of retinol. Disc electrophoresis on polyacrylamide gels revealed sizable incorporations of radioactivity from ^{14}C-retinol into the γ-globulin and albumin fractions. Moreover, when the amount of retinol binding was determined via the Sephadex technique, commercially prepared human γ-globulin and albumin bound similar quantities of ^{14}C-retinol. Assuming that RBP is present in serum of the order of 3–4 mg/100 ml, with a molecular weight of approximately 21,000, and that RBP binds retinol mole for mole (3), then the amount of retinol bound to serum in our experiments would be in excess of that required to completely saturate RBP. Thus, the additional incorporation of retinol into other serum fractions may reflect a mechanism whereby serum can transport an additional amount of retinol above that transported by RBP.

Of the vitamin A derivatives tested in our laboratory, ^{14}C-retinol was most strongly bound to human and rat serum, while ^{3}H-retinoic acid and ^{14}C-β-carotene were bound to a lesser degree. Several unlabeled derivatives inhibited the binding of retinol. These inhibitions may reflect a competition for binding sites. All-*trans* forms of retinal, retinoic acid, and retinyl acetate lowered the serum binding of retinol; the most effective was all-*trans* retinal. Interestingly, the 13-*cis* form of retinal had no appreciable effect on the binding process and could indicate that for binding to occur the ligand must possess an all-*trans* configuration. Kanai *et al.* (3) suggested that RBP might contain a thin cleft in which the flat retinol molecule would reside. It might also be possible that the binding of retinol to other serum proteins may involve similar clefts. If so, the nonplanar, 13-*cis* molecule might have difficulty in occupying such a cleft(s) and result in failure to bind to the proteins.

Our results emphasize the importance of future studies on the interaction of vitamin A with purified RBP, as well as with other purified serum proteins, for elucidating the

transporting mechanism of vitamin A in the blood.

Summary. A Sephadex G-25, batchwise, semimicro, partitioning technique is presented for investigating the binding of retinol to serum components. Rat sera from normal, vitamin A-deficient, and vitamin A-supplemented animals exhibited differences in the binding of retinol. These differences were related to variations in the serum content of vitamin A and protein. The major protein fraction responsible for binding retinol was the α-globulin. Other fractions, namely γ-globulin and albumin, were also capable of binding retinol but to a lesser degree. Human and rat sera exhibited a larger amount of binding for ^{14}C-retinol than for ^{3}H-retinoic acid and ^{14}C-β-carotene. Several unlabeled all-*trans* derivatives of vitamin A inhibited the binding of ^{14}C-retinol.

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