PROTEIN-BOUND RETINOL OR A RETINOL METABOLITE FOUND IN RAT AND HUMAN URINE

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SUMMARY

Rats injected with retinol-15-^{3}H were found, by fractionation of their urines on Sephadex G-50 or G-100, to excrete principally 2 labeled proteins: one of mol. weight > 100,000 (peak A), another of mol. weight 4600 (peak B). Both peaks showed U.V. light absorption at 330 nm. $CdCl_2$ poisoning of the rats resulted in a great increase in excretion of these proteins, vitamin A deficiency in a great decrease. Peak B had strong fluorescence (emission maximum at 426 nm). Attached radioactivity could not be extracted by heptane, chloroform-methanol, ether, butanol, even following pre-treatment with trichloroacetic acid (TCA), ethanol or 2M NaCl. Radioactivity from peak B was excluded by Sephadex G-15, even after TCA, ethanol or NaCl pre-treatment. A similar peak was detected in human urine. It is concluded that retinol or a retinol metabolite is excreted bound to a protein of mol. weight 4600.

Retinol binding protein (RBP) appears in human urine as a result of cadmium poisoning (1). We attempted to isolate holo-RBP (RBP with bound retinol) from rat urine following $CdCl_2$ administration, but were unable to find it. Instead, we isolated a protein of small molecular weight with retinol or a retinol metabolite bound to it. Some characteristics of this substance are here described.

The minimal dose of $CdCl_2$.2 1/2 H_2O which resulted in increased protein excretion in rat urine was .01 mg per 100 g body weight (Table I). However, significant increases in excretion of the protein-bound retinol or metabolite were not obtained until the dose was raised to .04 mg per 100 g given in 6 injections over 2 weeks. Fig. 1A shows fractionation of a 48 hour urine collection, after dialysis and lyophilization, on Sephadex G-100, from 2 rats injected with retinol-15-^{3}H. Two protein fractions were radioactive and absorbed U.V. light at 330 nm. A large increase (approx. 3-fold for peak B) in the protein and 330-absorbing material was seen after $CdCl_2$ poisoning (Fig. 1B).

In examining the protein-bound 330-absorbing material in urine

TABLE I

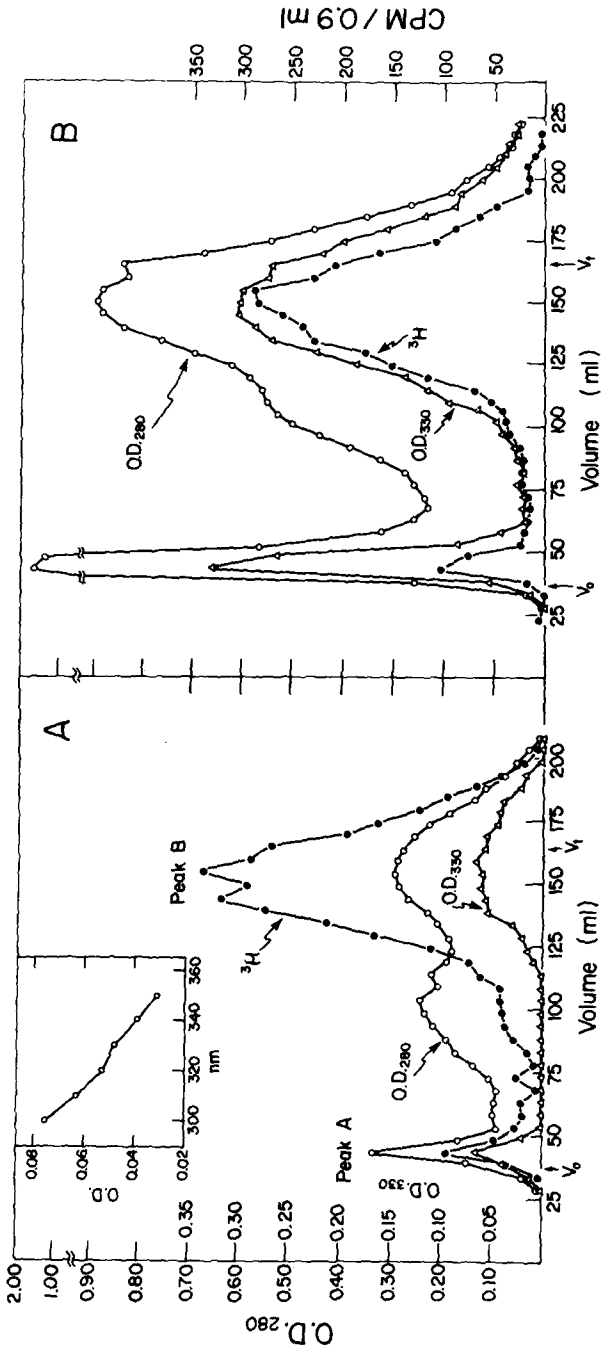
Urinary Protein Following a Single Injection of $CdCl_2$. 2 1/2 H_2O

Injected Amounts (mg/100 g body weight)	Total Protein in 24 hr. Urine Collection* Day 1 mg**	Day 2 mg	Day 3 mg	Day 4 mg
0 (saline)	135.6	139.4	161.0	178.5
.0025	168.1	134.0	138.8	67.2
.0050	128.5	198.0	115.5	173.6
.0100	227.0	267.0	281.0	288.0

* 3 rats/group
** protein assayed by the method of Lowry et al (6)

of vitamin A-deficient rats, it was found that both peaks had decreased sharply (3.7-fold for peak B), when compared to normal urine (Figs. 2A and B) (no labeled retinol was used in this experiment). It is of importance to note that a similar substance exists in normal human urine (Fig. 3A).

Fig. 1. Fractionation of urine from $CdCl_2$-treated rats on Sephadex G-100. Two rats (250 g each) of the Holtzman strain (Holtzman Co., Madison, Wis.) were injected intraperitoneally with retinol-15-^3H (100 µCi each; spec. activ. 1.24 Ci per mole, from New England Nuclear Corp., Boston, Mass.), suspended in aqueous medium (2) immediately before urine collection began (Fig. 1A). The injected retinol was specifically labeled at carbon 15, having been prepared by reduction of retinol with NaB^3H_4 (3). Its purity was checked by thin-layer chromatography on silica gel G (ethanol:cyclyhexane, 6:94): two impurities accounting for 6% of the radioactivity were present, with polarities lower than retinol. Three other rats (Fig. 1B) of the same weight had received 6 injections of 0.1 mg $CdCl_2$. 2 1/2 H_2O in saline intraperitoneally over 2 weeks, and then received retinol-^3H as the rats in Fig. 1A. Urines were collected for 48 hrs. in the dark in containers cooled in dry ice and protected by NaN_3 to prevent bacterial growth. Collected urines were pooled separately for the two experiments shown in Figs. 1A and 1B, assayed for creatinine (65 mg per cent in each), dialyzed overnight against 0.02 M phosphate buffer, pH 7.6 containing 0.2 M NaCl, lyophilized, dissolved in a small volume and fractionated in the above buffer solution on a Sephadex G-100 column (2.2 x 39.5 cm). Specific activity of peak B in Fig. 1A: 550 cpm per mg protein, 736 cpm per µg retinol equivalent (based on U.V. absorbance at 330 nm and $E^{1\%}_{1cm}$ = 1750). Peak B in Fig. 1B: 218 cpm per mg protein, 167 cpm per µg retinol equiv. Graphs are normalized with respect to creatinine. A correction was applied for tryptophan absorption at 330 nm, by determinating the ratio of absorbance at 330 and 280 nm for tryptophan. Radioactivity, ●___●; O.D. at 280 nm, o___o; O.D. at 330 nm, Δ___Δ . Inset: U.V. absorption spectrum of peak B.


A
B
Peak A
Peak B
O.D.280
O.D.330
^3H
Volume (ml)
CPM / 0.9 ml
O.D.
nm

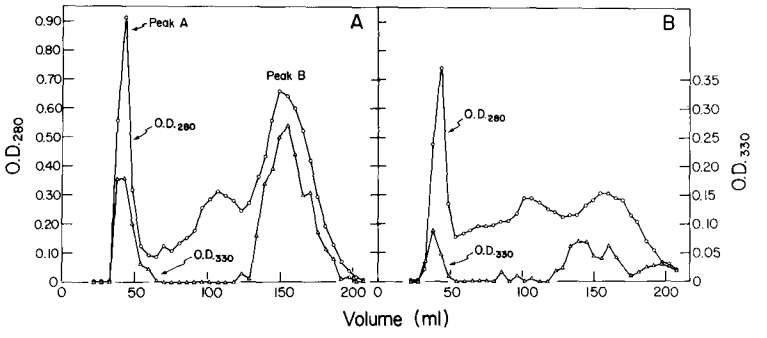


Fig. 2. Fractionation of urine from vitamin A-deficient rats on Sephadex G-100. Two rats (same strain as in Fig. 1) were made vitamin A-deficient as described (5). After they had lost 5% of their weight, their urines were collected for 24 hrs. and fractionated on Sephadex G-100 (Fig. 2B), exactly as described under Fig. 1. Urine from two pair-fed normal control rats (Fig. 2A) were used for comparison. Urine creatinine was 105 and 91 mg per cent for Figs. 2A and 2B, respectively. Graphs are normalized with respect to creatinine. A correction was applied for tryptophan absorption at 330 nm. O.D. at 280 nm, o___o; O.D. at 330 nm, Δ—Δ.

The protein peak eluted near the void-volume of the Sephadex G-100 column (peak A, Fig. 1A), which was radioactive after injection of labeled retinol and absorbed U.V. light at 330 nm, was at first thought to be the RBP-prealbumin complex (4). However, an attempt to split off the RBP by dialysis against .002 M Tris buffer (4) left the complex unchanged; it appeared in the void-volume of the column with absorption at 280 and 330 nm. No 330-absorbing material could be extracted from it with ether. Furthermore, its molecular weight was much higher than that of the RBP-prealbumin complex (4), to judge from its elution from the Sephadex G-100 column. It was, therefore, concluded that this peak was not RBP-prealbumin.

We next turned our attention to peak B, Fig. 1A. Its U.V. absorption spectrum (Fig. 1A, inset) showed a slight shoulder at 330 nm. Its fluorescence spectrum gave an emission maximum at 426 nm (excitation maximum at 346 nm). In comparing its fluorescence intensity to that of retinol in ethanol (excitation maximum at 337 nm, fluorescence maximum at 465 nm), it was found that, when U.V. absorbances at 330 nm were set equal, the protein-bound substance had a fluorescence intensity 12.3 times that of retinol.

Upon intraperitoneal injection of this fraction (peak B, Fig.

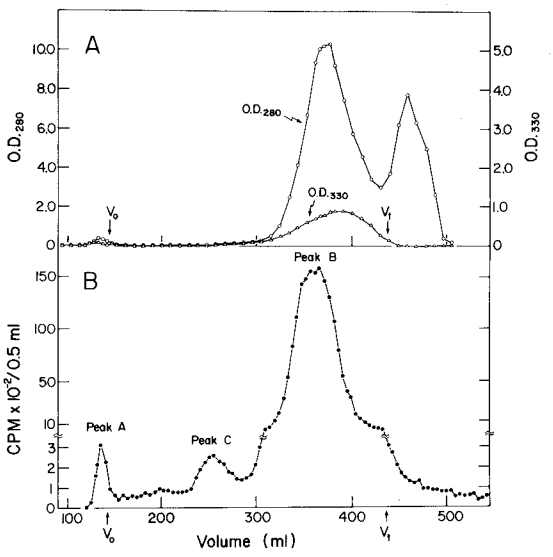


Fig. 3(A). Fractionation of urine from human subjects on Sephadex G-50. Fifty ml of mixed urine (22 mg per cent creatinine) from 3 normal male subjects was concentrated to a small volume by ultrafiltration (apparatus from Amicon Corp., Lexington, Mass.) through a membrane filter retaining molecules of molecular weight 1000 or higher. The concentrate was fractionated on a column of Sephadex G-50 (3.4 x 4.3 cm) in the buffer described under Fig. 1. A correction was applied for tryptophan at 330 nm.

Fig. 3(B). Two vitamin A-deficient rats (177 and 201 g) received a total of 300 μCi retinol-15-^{3}H each in 3 intraperitoneal injections over 3 days, as described in Fig. 1A. Urines were collected, concentrated and fractionated as described in Fig. 3A. K_{av} of peak B, 0.733, of peak C, 0.383. V_o, void-volume; V_t, total column volume. O.D. at 280 nm, o___o; O.D. at 330 nm, Δ___Δ.

1A) into 2 vitamin A-deficient rats for 3 days at the level of 8 μg retinol equivalents daily each (based on absorbance at 330 nm and an extinction coefficient $E^{1\%}_{1cm}$ = 1750), it failed to cure the animals of their deficiency.

When the solution corresponding to peak B, Fig. 1A, was shaken with heptane for 6 hours, no radioactivity, nor 330-absorbing, nor Carr-Price reactive material could be extracted. The radioactivity was not extractable into chloroform: methanol (2:1), ether or butanol. Pretreatment of the peak B solution with ethanol (brought to 66% ethanol) for 24 hrs., or trichloroacetic acid (TCA) (brought to 7%) for 2 hrs., or NaCl (brought to 2M), did not make the radioactivity extractable into ether. Following these treatments (ethanol, TCA, or NaCl), the labeled retinol or

metabolite was still attached to protein, since the label appeared in the void-volume upon fractionation on a Sephadex G-15 column. It contained sufficient protein to be measurable by the Lowry reaction (6).

To determine the molecular weight of the compound, raw urine from 2 rats injected with retinol-15-^{3}H was concentrated by ultra-filtration through a membrane filter holding back substances with molecular weight greater than 1000. This concentrate was then fractionated on Sephadex G-50. Fig. 3B displays the radioactive peaks obtained, showing the void-volume peak A (corresponding to peak A, Fig. 1), and peak B (corresponding to peak B, Fig. 1). Peak B from the Sephadex G-100 column (Fig. 1), when re-fractionated on Sephadex G-50, was shown to be eluted precisely as radioactive Peak B of Fig. 3B. A third small radioactive peak of molecular weight about 7000 was also found (peak C). By calibration of the column with cytochrome C, insulin and bacitracin, the molecular weight of the peak B complex was determined to be 4600.

To determine whether the excreted labeled substance was a true metabolite or merely retinol attached non-specifically to a urinary protein, we added labeled retinol to normal, unlabeled rat urine in amount identical (3.0 μCi) to that excreted after injecting 100 μCi retinol-15-^{3}H into another rat. After concentration of the urines in presence of unlabeled retinol and fractionation of the concentrates on Sephadex G-100 columns, the usual radioactive peak B was isolated from the rat which had been injected with retinol-^{3}H (Fig. 4A). Little radioactivity was eluted with this peak when taken from urine to which retinol-^{3}H had been added (Fig. 4B). When this experiment was repeated with ovalbumin added to normal, unlabeled urine, as well as retinol-^{3}H, no radioactivity was found attached to this protein after isolation on Sephadex G-100.

It is clear from the above data that the rat excretes a protein complexed, possibly covalently, with retinol or a metabolite thereof, with molecular weight of about 4600. It should be borne in mind that the ^{3}H-atom of the injected labeled retinol was in position 15, so that, if it is a metabolite, it cannot be retinoic acid. The possibility that it is retinol itself is made less likely by the shift to a lower wavelength of the fluorescence spectrum. On the other hand, the U.V. absorption spectrum

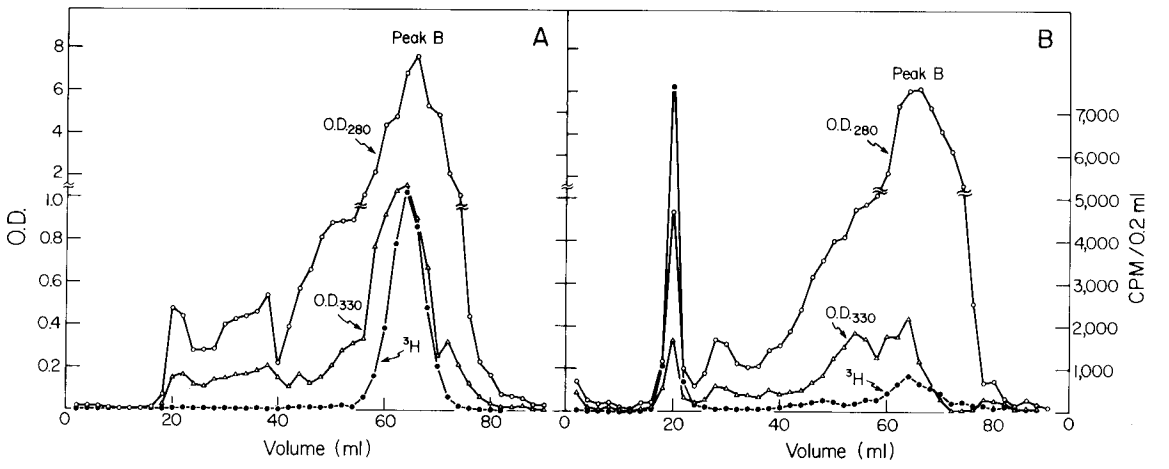


Fig. 4. Control experiment involving addition of labeled retinol to unlabeled urine. (A) Retinol-15-^{3}H (100 μCi) was injected into a normal rat as in Fig. 1, and urine was collected for 48 h as described (radioactivity, 3.0 μCi). It was concentrated to a small volume by ultrafiltration as in Fig. 3A, in presence of non-radioactive retinol (1 mg added dissolved in 0.1 ml ethanol). The concentrate was fractionated on a column of Sephadex G-100 (3.0 x 34.5 cm). Radioactivity in concentrate placed on column, 1.0 μCi; radioactivity in peak B, 0.92 μCi (6.5 μCi per mg protein).

(B) Retinol-15-^{3}H (3.0 μCi) was added to normal, unlabeled rat urine (48 h collection), which was then concentrated and fractionated on Sephadex G-100 as above in presence of unlabeled retinol. Radioactivity in concentrate placed on column, 1.1 μCi, radioactivity in peak B, 0.17 μCi (1.5 x 10^{-3} μCi per mg protein). Radioactivity, ●___●; O.D. at 280 nm, o__o; O.D. at 330 nm Δ____Δ .

showed a slight shoulder at 330 nm (Fig. 1A, inset). We cannot explain why the 330-peak is not more pronounced, if compared to that shown by RBP (7). The latter has a much larger amount of protein per mole of retinol, yet shows a clear peak at 330 nm besides the peak at 280 nm. One could postulate that in the compound we isolated, the 330 nm absorption is due to a metabolite with lower absorptivity than retinol; or that retinol is attached to a small fragment of RBP, containing most of the aromatic amino acids.

The large increase in excretion of the substance during $CdCl_2$ poisoning shows that, though some appears in normal urine (Fig. 1A), much of it is reabsorbed through the kidney tubules.

The appearance of a substance with similar U.V. absorption, fluorescence spectrum and molecular weight characteristics in human urine (Fig. 3A) is to be noted. We were unable to isolated holo-RBP from human urine, where it is normally found (1). It

may be contained in the small peak we observed (Fig. 3A) near the void-volume of the Sephadex G-50 column, where proteins of molecular weight of 20,000 or higher are eluted.

It has not escaped our notice that this substance may prove to be a useful indicator of vitamin A deficiency in man. It is easily isolated from urine, it has strong fluorescence, and evidence from the rat, at least, shows it to be responsive to vitamin A deficiency.

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