A SMALLER MOLECULAR WEIGHT RETINOL BINDING PROTEIN IN RAT TESTIS SEMINIFEROUS TUBULES

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SUMMARY

In the cytosol fraction in rat testis seminiferous tubules a lower molecular weight protein of ∿4,800 daltons that binds retinol with high specificity has been isolated and purified by ammonium sulfate precipitation and on Sephadex column chromatography. The hexane extract of the component gave a characteristic retinol fluorescence spectrum. The amino acid composition was qualitatively similar to the retinol binding protein in the blood with the exception that cystine and cysteine were absent.

It is well known that retinol is carried in the blood in combination with a specific protein (RBP) (1-3). What happens when RBP reaches a target tissue such as the testis is not clear. Whether RBP itself enters the tissue or free retinol diffuses into the tissue and then binds with another protein needs investigation. According to Bashor et al. (4) a specific tissue retinol binding protein component of ∿16,000 daltons is present in the cytosol of several rat tissues including the testis. In another study Ong and Chytil (5) isolated multiple retinol binding protein components in the rabbit lung. We have isolated and purified a low molecular weight protein component of ∿4,800 daltons in the cytosol of the seminiferous tubules (S.T.) of the rat testis that binds retinol with high specificity. The results of the study are reported here.

MATERIALS AND METHODS

All-trans - retinyl-11,12[$^{3}H_2$]-acetate with specific activity of 253 μCi/mg was a gift from Hoffman La Roche, Inc. All trans unlabeled retinyl acetate and retinol were purchased from Eastman Kodak. Purity of the compounds was

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assayed on an alumina column (6). Sephadex G-25 (coarse), G-75, G-200 and protein molecular weight determination kit were obtained from Pharmacia, and all other chemicals were of reagent grade.

Adult male Sprague-Dawley rats were used in this study. The animals were fed and watered ad libitum. They were killed by decapitation, and the testes were removed, cleaned and then decapsulated. From each testis the S.T. were separated mechanically (7) and washed twice with physiological saline. The washed S.T. were incubated in 40 ml of Hanks' physiological medium containing purified retinyl-11,12[$^3H_2$]-acetate (1.3 μCi/testis) predissolved in 0.5 ml of propylene glycol. To prevent oxidation 2 ml of 0.1% pyrogallol were added to each flask. The contents were incubated as described previously[2] at 37°C under a steady stream of nitrogen in a room with a red light. For each experiment the testes from 10 rats were used. After termination of the incubation, the S.T. were washed twice with 10 ml of 0.1% Triton X-100 to remove any adhering radioactivity on the outside membrane. The washed S.T. were then homogenized in 0.3 M sucrose buffered with 0.1 M KH_2PO_4 pH 7.4, passed through cheesecloth and the filtrate centrifuged at 9,000 x g for 20 minutes in a refrigerated centrifuge. The supernatant was centrifuged at 105,000 x g in a Beckman LS-45 ultracentrifuge to obtain the cytosol fraction.

The radioactivity in the aliquots was determined in a Beckman LS-255 scintillation counter after mixing in 12 ml of acquasol (New England Nuclear), and protein was determined by the procedure of Lowry et al. (8). The cytosol fraction was subjected to ammonium sulfate precipitation at different saturations of this salt. Precipitation was carried on at 0°C with pH between 7 and 7.4. The amount of ammonium sulfate required for various saturations was calculated from the table (9). The fraction was further purified by Sephadex G-200 column chromatography. Sephadex G-25 (coarse) was used for desalting the protein fractions.

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[2] Ahluwalia, B., Devi, A. and Clark, F. J. Fed. Amer. Soc. Exp. Biol. 32, 4093 (1973).

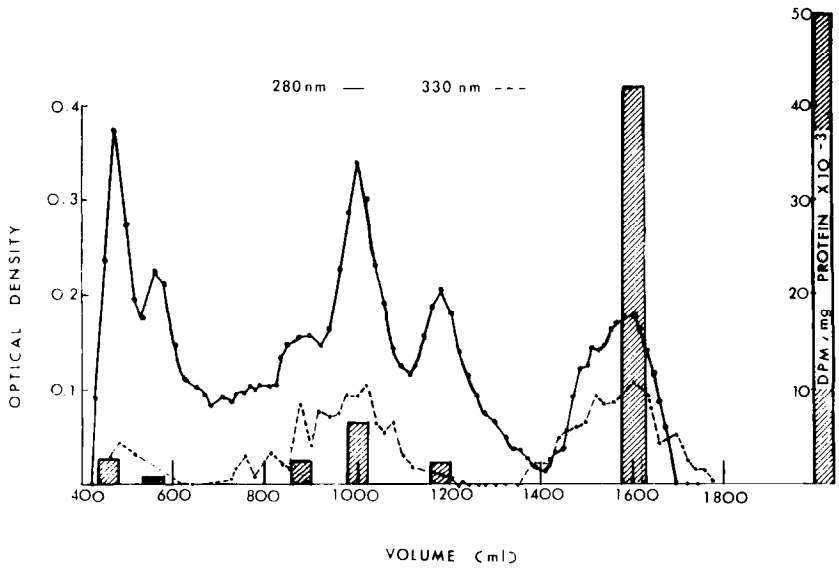

Fig. 1 Chromatography on Sephadex G-200 of fraction III obtained after precipitating retinyl-11,12-$^{3}H_2$-acetate-labeled cytosol fraction of rat testis seminiferous tubules with 50-100% ammonium sulfate saturation. The column (5 x 100 cm) was equilibrated with 0.05 M potassium phosphate buffer containing 0.1 M NaCl and 0.02% NaN_3. The sample applied to the column contained 50-70 mg of total protein in 10-15 ml buffer. Elution was carried out with the same buffer at the flow rate of 30 ml per hour at 4-8°C.

The molecular weight of the protein was determined by a gel-filtration technique (10) on a Sephadex G-75 column (2.6 x 70 cm). The column was first standardized with insulin (Sigma Chemical), RNase, chymotrypsmogen and ovalbumin proteins. A small amount of blue dextran polymer was chromatographed to determine void volume (V_o) of the column. After chromatography, the effluent volume (V_e) corresponding to the center of the peak of the eluted protein was measured and the values of V_e/V_o were plotted against the logs of their molecular weights.

The purity of the retinol binding protein component was determined by polyacrylamide gel electrophoresis by the systems of Reisfeld et al. (11) and Anker (12). Polymerization for polyacrylamide gel electrophoresis was induced by the addition of 0.1% ammonium persulfate in the presence of N,N,N´,N´-tetramethylethylenediamine, and gels were stained for at least one hour in a

0.1% solution of aniline blue black in 10% trichloroacetic acid followed by destaining in 7% acetic acid.

Amino acid analyses of the component were carried out in an evacuated sealed tube at 110°C for 22 hours with a Beckman 120B amino acid analyzer.

RESULTS AND DISCUSSION

The cytosol fraction was subjected to ammonium sulfate precipitation at three different saturations: 0-15% (fraction I), 15-50% (fraction II) and 50-100% (fraction III). Fractions I (3597 DPM/mg protein) and III (5955 DPM/mg protein) were of higher specificity than fraction II (912 DPM/mg protein). Fraction I was found to be insoluble in phosphate buffer with 0.01-1.0 M salt concentration at pH 7.4; fraction III was soluble and was fractionated on a Sephadex G-200 column using a 0.05 M phosphate buffer containing 0.1 M NaCl and 0.02% NaN_3 at pH 7.4, at 4-8°C. The elution pattern is shown in Fig. 1. The isolation and purification procedure is summarized

TABLE I

Fractionation of Labeled Seminiferous Tubules[1]

Type of fraction[2]	DPM/mg Protein	Total Protein (mg)	Percentage of Total Protein of Homogenate
Homogenate	9,776	1,026	100.0
Cytosol	4,444	423	41.2
0-15% $(NH_4)_2SO_4$ fraction I	3,597	18	1.8
15-50% $(NH_4)_2SO_4$ fraction II	912	219	21.3
50-100% $(NH_4)_2SO_4$ fraction III	5,955	123	12.0
Sephadex G-200 column	42,735	0.25-0.5	.025-0.05

[1] The seminiferous tubules from 50 rats were labeled with retinyl-11, 12-[$^3H_2$]-acetate (1.3 µCi/testis) under the experimental conditions described in the text.

[2] Fractions obtained after differential centrifugation. For details see text.

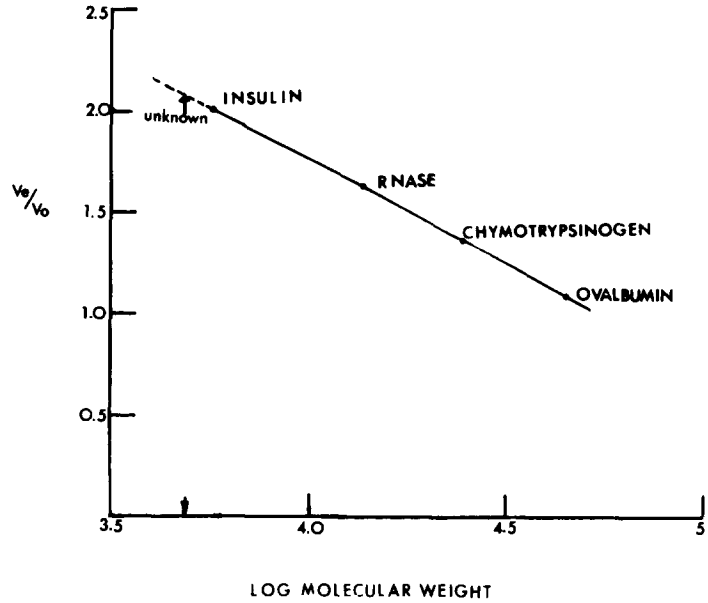

Fig. 2 Estimation of molecular weight by gel filtration. A portion of the purified protein was dissolved in 0.05 M potassium phosphate buffer, pH 7.4, 0.1 M NaCl and 0.02% NaN_3 and chromatographed on Sephadex G-75 column (2.6 x 70). Elution with the same buffer was carried out at a flow rate of 40 ml per hour at 4-8°C. Fractions of 5 ml each were collected. Elution was monitored at 230 nm.

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in Table I. The hexane extract of peaks revealed that retinol was concentrated in the last peak - 8.6 µg/mg protein - compared with less than 1.0 µg/mg protein in the other peaks. In an experiment where the cytosol was incubated with non-labeled retinyl acetate and then fractionated on a G-200 column, the elution pattern was similar to the one obtained upon incubation with labeled retinyl acetate (Fig. 1). The peak containing the highest level of retinyl acetate corresponded to the peak from the cytosol isolated following incubation with [^{3}H]-retinyl acetate. These results suggest that the last peak in Fig. 1 contains a component that binds retinol with high specificity. This peak showed a single band in analytical polyacrylamide electrophoresis at pH 3.5 and contained almost 50 to 55% of the radioactivity applied to the gel.

The molecular weight of the protein was found to be ∿4,800 daltons by the gel filtration technique (Fig. 2).

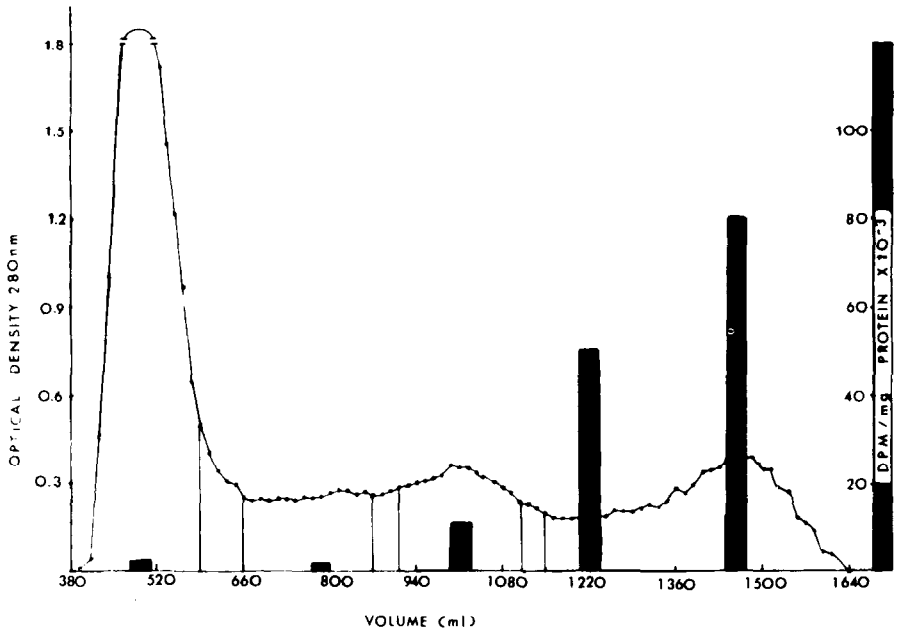

Fig. 3 Elution profile of cytosol fraction obtained after labeling seminiferous tubules with retinyl-11,12[$^3H_2$]-acetate. The Sephadex G-200 column (5 x 100 cm) was equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, 0.1 M NaCl and 0.02% NaN_3. Elution was carried out under the same experimental conditions as in Fig. 1.

In another study the cytosol fraction following incubation of the S.T. with labeled retinyl acetate was not subjected to ammonium sulfate precipitation but instead was freeze-dried and fractionated directly on the Sephadex G-200 column. Two peaks of molecular weights ∿4,800 and ∿16,000 were observed (Fig. 3) suggesting that in the S.T. cytosol there are at least two retinol binding components of different molecular weights. These results do not provide any definitive answer as to whether the lower molecular weight component reported here is a part of a bigger molecule or there are two individual components. It has been reported that in the rabbit lung three components of molecular weights between ∿14,000 and ∿17,000 that bind retinol with high specificity are present.

The amino acid composition of the component showed the absence of cystine and cysteine (Table II). The smaller size and the amino acid composition

TABLE II

Amino Acid Composition of Retinol Binding Component
Isolated from Cytosol of Rat Testis Seminiferous Tubules

Amino Acid	Percentage Distribution[1]	Estimated Number of Residues[2]
Lysine	6.87	3
Histidine	1.57	1
Arginine	2.40	1
Aspartic acid	16.43	7
Threonine	5.26	2
Serine	6.97	3
Glutamic acid	18.53	8
Proline	8.19	3
Glycine	11.92	5
Alanine	7.14	3
1/2 cystine	0	0
Valine	4.59	2
Methionine	0.84	Trace
Isoleucine	3.50	2
Leucine	4.60	2
Tyrosine	0.97	1
Phenylalanine	1.98	1
Tryptophan	N.D.[3]	N.D.
TOTAL[4]		44

[1] Average of two determinations.

[2] Calculated on the basis of 1.0 residue of histidine per molecule.

[3] Not determined.

[4] Excluding ammonia and tryptophan.

suggest that this component might have a very loose structure which enables it to bind retinol with high affinity.

The function of the lower and higher molecular weight components is not known. The possibility that the lower molecular weight component might be a partial degradation of a single component cannot be excluded from these studies. Bashor *et al.* (4) postulated that the tissue cytosol retinol binding component is analogous to steroid hormone receptor proteins; however the smaller size of the tissue retinol binding components compared to steroid receptor proteins

and our findings of the presence of retinol in bovine sperm acrosomes[3] raises the possibility that the tissue retinol binding component may not have a role similar to that of the hormone receptor proteins (13). It has been reported that rat serum RBP (1) is of ∿20,000 molecular weight and is associated with pre-albumin to form a complex of ∿70,000 molecular weight. It would be of interest to investigate the relationship of RBP to the tissue-binding component(s) in the testis.

Our preliminary study shows that compared with retinol this component has only one tenth of the binding affinity for retinoic acid. This may explain the fact that at least in the testis the absence of a specific binding component for retinoic acid may render this compound biologically ineffective. Our study has shown that retinoic acid implanted locally in the testis was ineffective in restoring the germinal epithelium to normal in vitamin A deficient rats (14).

ACKNOWLEDGMENT

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REFERENCES

1. Muto, Y. and Goodman, D. S., J. Biol. Chem. 247, 2533-2544 (1972).
2. Kanai, M., Raz, A. and Goodman, D. S., J. Clin. Invest. 47, 2025-2044 (1968).
3. Peterson, P. A. and Berggard, I., J. Biol. Chem. 246, 25-33 (1971).
4. Bashor, M. M., Toft, D. O. and Chytil, F., Proc. Nat'l. Acad. Sci. USA 70, 3483-3487 (1973).
5. Ong, D. E. and Chytil, F., Biochem. Biophys. Res. Commun. 59, 221-229 (1974).
6. Futterman, S. and Andrews, J. S., J. Biol. Chem. 239, 81-84 (1964).
7. Hall, P. F., Irby, D. C. and de Krester, D. M., Endocrinology 84, 488-496 (1969).
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265-275 (1951).
9. Green, A. A. and Hughes, W. L., in Methods in Enzymology, vol. 1, Colowick, S. P. and Kaplan, N. O., eds, Academic Press, pp. 67-78 (1955).
10. Whitaker, J. R., Anal. Chem. 35, 1950-1953 (1963).
11. Reisfeld, R. A., Lewis, U. J. and Williams, D. E., Nature 195, 281-283 (1962).
12. Anker, H. S., Fed. Eur. Biochem. Soc. Lett. 7, 293 (1970).
13. Sherman, M. R., Corvol, P. L. and O'Malley, B. W., J. Biol. Chem. 245, 6085-6096 (1970).
14. Ahluwalia, B. and Bieri, J. G., J. Nutr. 101, 141-152 (1971).

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[3] Gambhir, K. K. and Ahluwalia, B. J. Reprod. Fert. (in press).