[CANCER RESEARCH 42, 1033–1037, March 1982]
0008-5472/82/0042-0000$02.00

Purification and Partial Characterization of Cellular Retinol-binding Protein from Human Liver[1]

David E. Ong

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

ABSTRACT

Cellular retinol-binding protein (CRBP) has been purified to homogeneity from normal human liver. The procedures in the purification involved primarily gel filtration and ion exchange chromatography, resulting in a 3000-fold purification with greater than 40% yield. The protein is a single polypeptide chain with molecular weight of 14,800. The protein binds retinol in a manner which considerably alters its spectrum from that observed in organic solution. Many of the properties of human CRBP including molecular weight, amino acid composition, and spectrum of bound retinol are similar to those observed previously for rat CRBP. The availability of pure human CRBP should aid in elucidating its role in the action of retinol and also in more easily monitoring the considerable changes in level of this protein reported in some human cancers.

INTRODUCTION

Vitamin A is known to be required for the proper development and differentiation of many tissues (27). In addition, the presence or absence of compounds of the vitamin A family (retinoids) such as retinol, retinoic acid, and analogs of these compounds appears to affect the appearance and progression of various induced or spontaneous neoplasias in experimental animals as well as humans (6, 25).

Two intracellular proteins which bind vitamin A-like compounds with high affinity and specificity are present in many tissues of the body (3). The first binds retinol and is called CRBP[2] (1, 17). It has been purified from several rat, bovine, and dog tissues (12, 17, 18, 22, 23). The second binds retinoic acid and is called cellular retinoic acid-binding protein (15, 18). These proteins have been implicated in the molecular action of retinol and retinoic acid (2, 16). In addition, the levels of these proteins frequently show striking changes in cancerous tissue compared to normal tissue (4, 21). CRBP underwent a considerable elevation in level in chemically induced colorectal adenocarcinoma of rat (20). In humans, epidermoid carcinomas of the oral cavity (19) and some breast tumors (5) have been shown to have significant elevations of CRBP compared to normal tissue. In order to study the mechanisms of these elevations as well as to compare the CRBP in normal human tissue to that in cancerous tissue, it will be necessary to have purified preparations.

Here, I report the purification and partial characterization of this protein from normal human liver.

MATERIALS AND METHODS

Materials

Sephadex G-75 and molecular weight calibration proteins (ovalbumin, chymotrypsinogen A, RNase A) were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Myoglobin (whale) was from Sigma Chemical Co., St. Louis, Mo. The molecular weight calibration kit (prestained) for SDS-polyacrylamide gel electrophoresis was purchased from Bethesda Research Laboratory, Inc., Gaithersburg, Md. DEAE-cellulose (DE52)- and CM-cellulose (CM52) were products of Whatman, Inc., Clifton, N. J. All-*trans*-[15-^{3}H]retinol (15 Ci/mmol) was synthesized and purified as described previously (11). The human liver sample was obtained from the Surgical Pathology Laboratory, Vanderbilt University Hospital, through the cooperation of Dr. David Page.

Methods

Detection and Quantitation of CRBP. The binding protein was quantitated by incubation of samples with [^{3}H]retinol followed by sucrose gradient centrifugation as described previously (20). Briefly, samples were diluted to 0.3 ml with Tris-HCl buffer, pH 7.5, and then incubated at 4° for 4 hr with 30 pmol [^{3}H]retinol. Parallel incubations contained a 100-fold excess of unlabeled retinol. Immediately prior to sucrose gradient centrifugation, the incubations were treated with 0.2 ml of dextran-coated charcoal suspension to remove free ligand. Aliquots (0.2 ml) of the incubation mixtures were submitted to centrifugation on linear 5 to 20% (w/v) sucrose gradients in 0.05 M Tris-HCl, pH 7.5. The binding protein was revealed in the radioactivity profile of the fractionated gradient as a peak of radioactivity in the 2S region which was abolished in the presence of excess unlabeled ligand. The radioactivity in the 2S peak (specific binding) was used to quantitate the amount of binding protein present, assuming one binding site per molecule of binding protein. The detection of CRBP during column chromatography was accomplished by either monitoring fluorescence of bound retinol (excitation, 350 nm; emission, 475 nm) or monitoring its absorbance (λ_{max}, 350 nm).

Preparation of Liver Extract and First Steps in Purification. All steps were carried out at 0–4°. Human liver (total, 800 g) was homogenized in 3 volumes (w/v) of 0.01 M Tris-HCl, pH 7.5, for 60 sec in a Waring Blendor. The homogenate was centrifuged at 15,000 × *g* for 15 min to remove cell debris. A small portion of the resulting supernatant was subjected to centrifugation at 100,000 × *g* for 1 hr, and the supernatant liquid was collected for determination of total soluble protein and quantitation of CRBP. To the remaining preparation was added glacial acetic acid, dropwise, until pH 5 was reached. Precipitated cellular organelles and protein were removed by centrifugation at 20,000 × *g* for 15 min. To the supernatant liquid (2200 ml) was added CM52 CM-cellulose (settled volume, 220 ml) equilibrated previously at pH 5.0 in 0.01 M sodium acetate buffer. The suspension was stirred for 30 min before the CM-cellulose was removed by filtering through a sintered glass funnel. The filtrate was adjusted to pH 7.5 with 6 N NaOH. The solution was then lyophilized to dryness.

Column Chromatography. All steps were carried out at 0–4°. The lyophilized material was dissolved in 200 ml distilled water and dialyzed

[1] This work was supported by USPHS Grant CA-20950.

[2] The abbreviations used are: CRBP, cellular retinol-binding protein; SDS, sodium dodecyl sulfate; CM, carboxymethyl; RBP, serum retinol-binding protein.

Received July 21, 1981; accepted November 30, 1981.

against 0.2 M NaCl-0.05 M Tris-HCl, pH 7.5. Fifty-ml portions were submitted to gel filtration on columns (5 × 75 cm) of Sephadex G-75 after the addition of 1 μmol retinol in 0.1 ml isopropanol. The column was equilibrated and run with the same composition buffer as used for dialysis. The elution was monitored by following the fluorescence of the bound retinol. Fractions containing CRBP were combined from the 4 separate runs required to process all material and concentrated by ultrafiltration to 40 ml. This solution, after dialysis against 0.01 M Tris-acetate, pH 8.3, and addition of retinol as above, was divided into 2 equal portions and applied to 2 similar columns (2.6 × 20 cm) of DEAE-cellulose (DE52) equilibrated with the above buffer. After loading, the columns were washed with buffer until 20 fractions of 6.5 ml had been collected. The columns were then eluted with a linear gradient of the same buffer from 0.01 to 0.33 M (total volume, 550 ml). The column effluent was monitored for absorbance at 280 and 350 nm. Fractions containing CRBP, as revealed by an absorbance peak with λ_{max} 350 nm, were combined. After dialysis against 0.01 M Tris-acetate, pH 8.3, the preparation was applied to a similar but smaller DEAE-cellulose column (1.6 × 18 cm) and eluted with a linear gradient of Tris acetate buffer, pH 8.3, from 0.01 to 0.3 M in a total volume of 550 ml. Fractions containing the binding protein were detected by the absorbance at 350 nm and combined, and the volume was reduced by ultrafiltration to 10 ml. The sample was dialyzed against 0.01 M imidazole acetate, pH 6.4; 1 μmol retinol in 0.1 ml isopropanol was added; and the solution applied to a small (1.6 × 15 cm) column of DEAE-cellulose (DE52) prepared and equilibrated in the imidazole buffer. The column was eluted with a linear gradient of imidazole acetate from 0.01 to 0.10 M at pH 6.4. Fractions containing the binding protein were combined and concentrated by ultrafiltration to 7 ml. The solution was frozen in 1-ml aliquots for later analyses.

Gel Electrophoresis. Purity was assessed by gel electrophoresis in 2 systems. The first was a 15% polyacrylamide disc gel system, pH 8.9, as described by Maurer (13) with the exception that the stacking gel was replaced by Sephadex G-75 in stacking gel buffer. The second system was SDS-polyacrylamide electrophoresis as described by Weber and Osborn (26).

Molecular Weight Determination. The molecular weight of the binding protein was estimated by gel filtration on a standardized column of Sephadex G-75 as described previously (17). The elution position was determined by monitoring fluorescence of bound retinol.

The molecular weight was also estimated by SDS-polyacrylamide electrophoresis on 11% polyacrylamide slab gels (10) using prestained protein molecular weight standards in the range of 3000 to 43,000.

Amino Acid Analyses. The amino acid composition of human CRBP was determined commercially by AAA Laboratory, Murcer Island, Wash. Cysteine was determined as cysteic acid, and methionine was determined as the sulfone after low-temperature performic acid oxidation. Values for serine and threonine were corrected for presumed degradation of 10 and 5%, respectively, as suggested by AAA Laboratory.

Determination of Molar Extinction Coefficient of Bound Retinol. Aliquots (25 μl) of solutions of CRBP-retinol with A_{350} in the range of 0.2 to 0.6 absorbance units were added to 50 μl ethanol with mixing. Then, 2 ml of hexane were added, and the resulting mixture was mixed vigorously for 30 sec. The lower water phase was allowed to settle, and the fluorescence (excitation, 325; emission, 460) of the hexane solution was determined. The amount of retinol present was then determined from a standard curve of fluorescence *versus* retinol concentration, established previously. The concentration of stock solutions of retinol was calculated from the A_{325} of ethanol solutions of retinol, using a molar extinction coefficient of 52,000 for all-*trans*-retinol in ethanol (14).

Other Procedures. Fluorescence measurements and spectra were determined with an Amicon-Bowman spectrophotofluorometer. Absorbance and adsorption spectra were determined with a GCA/McPherson Model EU-721-D spectrophotometer.

RESULTS AND DISCUSSION

In the sample of human liver chosen for the purification, CRBP represented about 0.033% of the total soluble protein. The liver sample of 800 g contained approximately 14 mg CRBP. Table 1 presents the steps of purification leading to a homogeneous preparation of CRBP. These steps, described in detail in "Materials and Methods," are discussed briefly here.

After the liver was homogenized in 3 volumes of buffer, debris was removed by a low-speed centrifugation. The resulting cloudy supernatant liquid was then adjusted to pH 5. This caused the precipitation of considerable material including all cell organelles. After the precipitated material was removed by centrifugation, the resulting clear supernatant liquid was considered the starting material for the purification, as expressed in Table 1. Treating this solution batchwise with CM-cellulose removed a considerable amount of protein, the CRBP remaining unbound, effecting a 3-fold purification at this point. To aid in monitoring the purification, sufficient retinol was added to saturate the protein. This addition of retinol was repeated before each succeeding step in an attempt to keep the protein fully saturated.

The material obtained after the CM-cellulose step was then submitted to gel filtration on Sephadex G-75. Four runs were required to process the total sample. The elution of CRBP was followed by monitoring the fluorescence of bound retinol (excitation, 350 nm; emission, 475 nm). A prominent peak of fluorescence was observed, centered at an elution volume of 1125 ml. The fractions containing this peak were combined (total volume, 180 ml). The total volume of the column used was 1600 ml. The amount of fluorescence observed was considerably greater than expected, based on CRBP content as assessed by sucrose gradient centrifugation and assuming the fluorescence yield is similar to that observed previously for the rat CRBP-retinol complex (17). A reason for this apparent discrepancy was suggested in the next step, chromatography on a DEAE-cellulose column at pH 8.3 (Chart 1). When absorbance was monitored at 350 nm, chosen because that is the λ_{max} for retinol bound to rat CRBP, 2 peaks were observed. The first was centered at 68 ml (Fraction 35), and the second was at 480 ml (Fractions 93 to 94) of the 550-ml gradient used to develop the column. When the absorption spectrum of the first peak was examined in the 300- to 400-nm range, it was found to have an absorbance peak with λ_{max} at 330 nm. This part of its spectrum closely resembled that reported for human RBP (9). Since RBP is synthesized in liver (24), this early eluting material may well be this protein. The absorption spec-

Table 1

Steps in the purification of CRBP from human liver

Step	Total protein[a] (mg)	CRBP (mg)	Purification (-fold)	Recovery (%)
pH 5 supernatant	31,800	14.0[b]		
CM-cellulose supernatant	9,700	12.8[b]	3	91
Sephadex G-75	600	11.0[b]	42	78
DEAE-cellulose, pH 8.3	36	9.2[b]	580	66
DEAE-cellulose, pH 8.3 (repeat)	15	8.3[b]	1,050	59
DEAE-cellulose, pH 6.4	5.7[d]	5.7[c]	2,270	41

[a] Estimated by microbiuret assay (8).
[b] Estimated by sucrose gradient centrifugation binding assay (21).
[c] Determined by A_{350}, using ϵ = 59,200 and a molecular weight of 14,800 for the binding protein (see text).
[d] Assumed since no protein other than CRBP was demonstrable.

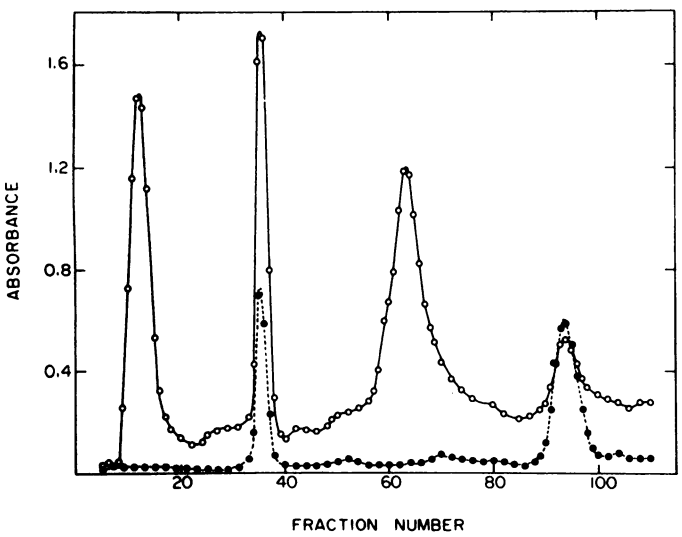

Chart 1. Chromatography of CRBP on DEAE-cellulose at pH 8.3. A Tris-acetate gradient (total volume, 550 ml) from 0.01 to 0.33 M was begun at Fraction 21. Fraction size was 6.5 ml. Absorbance was monitored at 280 nm (○) and 350 (●).

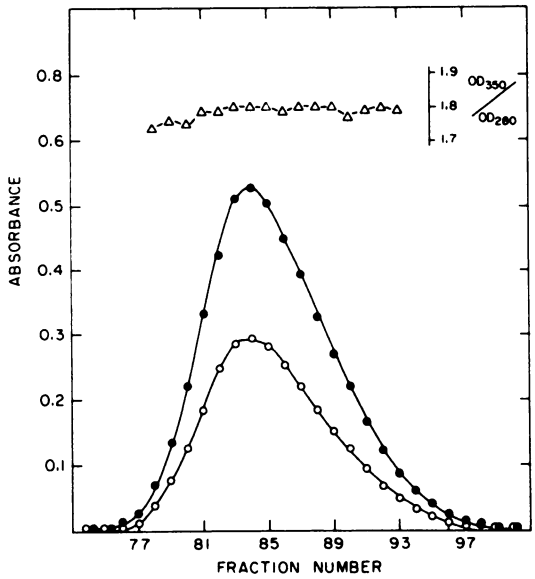

Chart 2. Chromatography of CRBP on DEAE-cellulose at pH 6.4. An imidazole acetate gradient from 0.01 to 0.1 M (total volume, 500 ml) was begun at Fraction 6. Fraction size was 5.0 ml. Absorbance (*OD*) was monitored at 280 (○) and 350 (●) nm. Ratio of A_{350} to A_{280} (△).

trum of the second peak was considerably different from the first peak, closely resembling the spectrum observed previously for rat CRBP with λ_{max} at 350 nm (17). Since CRBP and RBP are of somewhat similar size (M.W. 14,800 *versus* M.W. 21,000), the fluorescence of bound retinol in the Sephadex G-75 elution was probably due to overlap of the peaks from each retinol complex. Under the conditions used for the Sephadex G-75 column runs, proteins of this similarity in size were not well resolved. However, the assay of CRBP by sucrose gradient centrifugation must not be significantly perturbed by the presence of RBP, since the amounts calculated were consistent throughout the purification, particularly before and after this column step which separated the 2 proteins. Evidently, the conditions used do not allow the detection of RBP also. The reason(s) for this is unknown.

Fractions comprising the second peak, assumed to be CRBP, were combined (2 column runs were carried out) and submitted to chromatography on a similar but smaller column of DEAE-cellulose, again at pH 8.3. On this column, the CRBP, monitored by absorbance at 350 nm, eluted in a peak centered at 305 ml of the 550-ml gradient (not shown). The absorbance at 350 nm of the combined sample was now slightly greater than the absorbance at 280 nm, consistent with the calculation that CRBP comprised about 50% of the total protein (Table 1) at this step.

The final step was chromatography on DEAE-cellulose at pH 6.4, shown in Chart 2. CRBP eluted in an almost symmetrical peak centered at 395 ml (Fraction 84) of the 550-ml gradient used to develop the column. It appeared that the preparation was homogeneous as the ratio of A_{350} (bound retinol) to A_{280} (protein) showed little variation over the peak (Chart 2).

The purity of the combined material was then assessed by disc gel electrophoresis. The position of CRBP on the gel was determined after electrophoresis by illuminating the gel with long-wave UV light in the dark. A single yellow-green band of fluorescence was observed. Its position corresponded exactly to the single protein band revealed after fixing and staining, shown in Fig. 1. The increased staining density near the top of

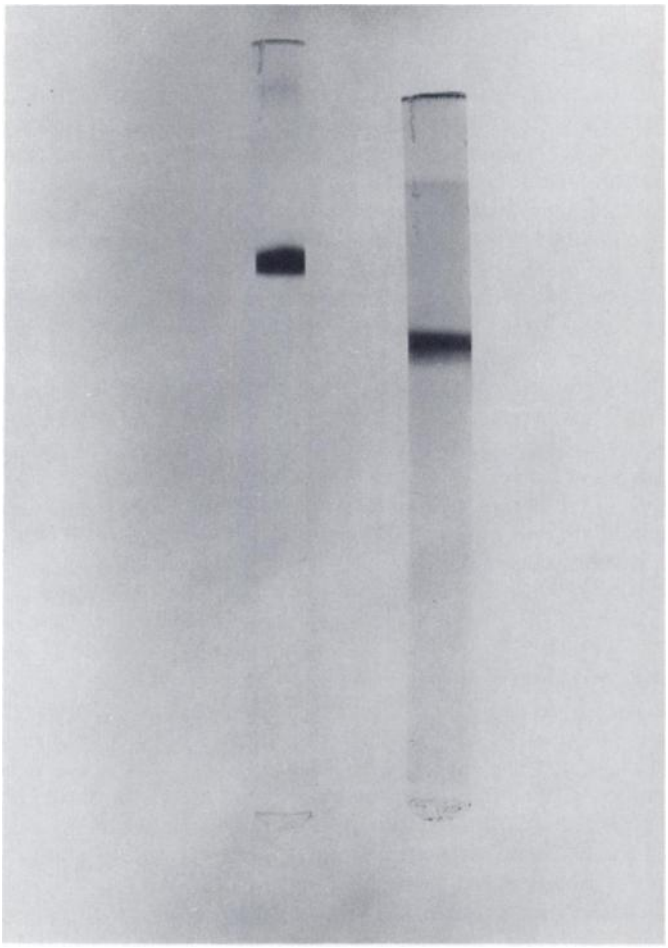

Fig. 1. CRBP after polyacrylamide gel electrophoresis in the system using SDS (*left*) and the disc gel system (*right*). About 10 μg of protein were applied to each gel. Staining was with Coomassie blue.

Table 2
Amino acid composition of human liver CRBP

Amino acid	mol/100 mol	No. of residues	No. of residues (rat)[a]
Alanine	3.52	5	4
Arginine	5.13	7	8
Aspartic acid	15.15	20	18
Glutamic acid	14.00	19	19
Glycine	8.36	11	10
Cysteine	2.27	3	3
Histidine	0.94	1	3
Isoleucine	4.58	6	7
Leucine	8.67	11	11
Lysine	7.92	10	11
Methionine	3.34	4	5
Phenylalanine	5.01	7	6
Proline	2.13	3	2
Serine	2.92	4	3
Threonine	5.67	7–8	7
Tyrosine	1.30	2	3
Valine	9.11	12	10
Tryptophan	ND[b]	4[c]	4
	100.0	136–137	134

[a] Taken from primary sequence[3] with asparagine + aspartic acid = aspartic acid and glutamine + glutamic acid = glutamic acid.
[b] ND, not determined.
[c] Assumed from rat CRBP data.

the gel is an artifact generated by using Sephadex G-75 in place of a stacking gel. The material was also examined by SDS electrophoresis. One major band was observed (Fig. 1) with a very faint amount of staining near the top of the gel. Based on these gels, the preparation was judged to be virtually homogeneous. The purification accomplished was about 3000-fold, based on total soluble liver protein. The yield was greater than 40%. The material from this step was used for the remainder of the studies presented here.

Molecular Weight Determination. The molecular weight of human CRBP was estimated by 2 methods. A sample was submitted to gel filtration on a calibrated Sephadex G-75 column. The elution position was monitored by following the fluorescence of bound retinol and was equivalent to a molecular weight of 14,500.

The molecular weight was also estimated by SDS-polyacrylamide electrophoresis by the procedure of Laemmli (10). The migration distance was consistent with a molecular weight of 15,200. A molecular weight average of 14,800 was thus used for the purposes of calculations in this report. The agreement between these methods is consistent with human CRBP being a single polypeptide chain.

Amino Acid Composition. The amino acid composition of human CRBP except for tryptophan is presented in Table 2. The data are presented as mol of each residue per 100 residue mol. Interestingly, by assuming the number of cysteine residues to be 3 and normalizing the other residue data to this assumption, an amino acid composition expressed as number of residues per CRBP is generated (Table 2, Column 2) which is quite similar to the known amino acid composition of rat CRBP. Those data were taken from the primary structure of rat liver CRBP[3] and suggest that there may be considerable similarity in the proteins from these 2 sources.

Absorption Spectrum. The absorption spectrum of purified human CRBP complexed with retinol is shown in Chart 3. As has been noted previously for CRBP from other species, the spectrum is dominated by the absorbance of bound retinol with λ_{max} at 350 nm (17, 23). This spectrum of retinol is considerably altered from its spectrum in organic solvents, having λ_{max} red shifted 25 nm, and with the introduction of fine structure as shown by the shoulder at 330 nm and the second peak with λ_{max} at 368 nm. As reported previously, this altered spectrum is quite likely due to the all-*trans*-retinol being bound in a planar configuration. A planar configuration is apparently prevented by steric hindrance when retinol is free in solutions of organic solvents (17).

The protein absorbance peaks at 284 nm and also shows a definite second peak at 292 nm due to tryptophan. The highest ratio of A_{350} to A_{280} observed for the CRBP-retinol complex was 1.8 and was presumed to represent CRBP fully saturated with retinol.

Extinction Coefficients. The extinction coefficient of retinol bound to CRBP was estimated by extracting aliquots of solution of retinol-CRBP of known A_{350} with organic solvent. The amount of retinol in the extract was then determined by comparing the fluorescence of the extract to the fluorescence of standard solutions of all-*trans*-retinol. The fluorescence spectra of the extracts were identical to those of the standard retinol solutions. Assuming 100% efficiency of extraction and ϵ of 52,000 for retinol in ethanol (14), the molar extinction coefficient of retinol bound to human CRBP was 59,200 ± 250 (S.E.) at λ_{max}, 350 nm. Based on the ratio of A_{350} to A_{280} of 1.8, the ϵ_{280} would be 32,900 for CRBP saturated with retinol.

Fluorescence Spectra. As was observed previously for rat CRBP (17) and for other proteins that bind retinol, such as β-lactoglobulin and RBP (7), there is considerable enhancement of the retinol fluorescence when it binds to human CRBP compared to its fluorescence in a solvent such as ethanol. This increase in intensity is about 7- to 8-fold. The fluorescence

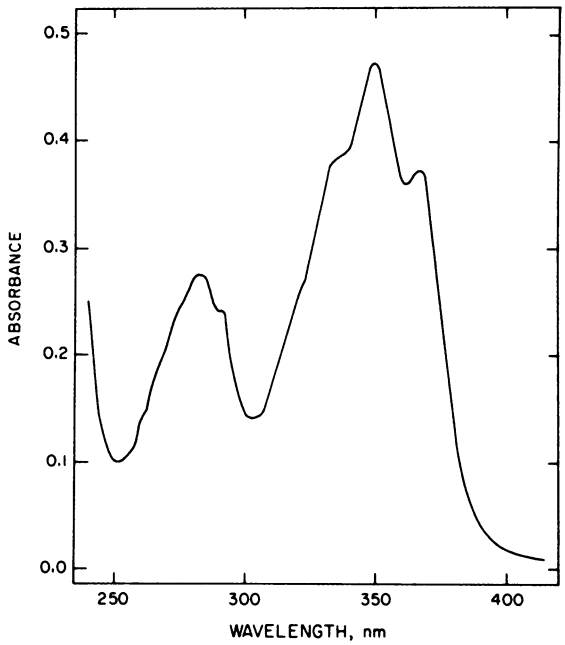

Chart 3. Absorption spectrum of CRBP with bound retinol, 8.0 × 10^{-6} M, in 0.05 M imidazole acetate, pH 6.4.

[3] L. Rask and P. Peterson (University of Uppsala, Sweden), personal communication.

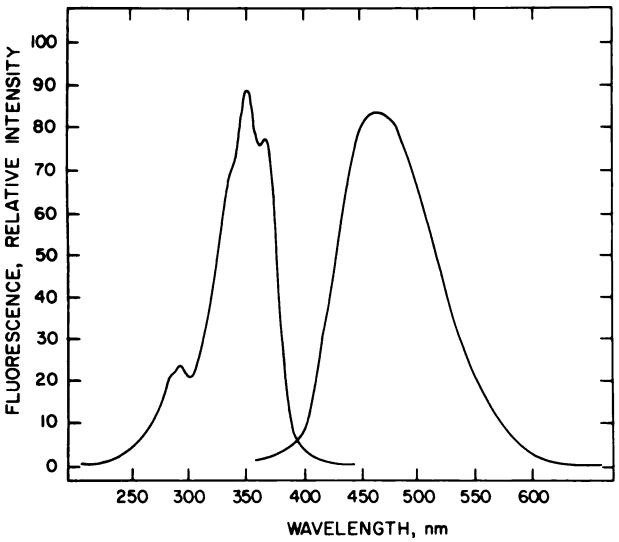

Chart 4. Uncorrected fluorescence spectra of retinol bound to CRBP, 2.8 × 10^{-6} M, in 0.05 M imidazole acetate, pH 6.4. Excitation spectrum (*left*) was determined by monitoring emission at 470 nm. Emission spectrum (*right*) was determined with excitation at 350 nm.

excitation and emission spectra of the bound retinol are shown in Chart 4. Both spectra are uncorrected. The excitation spectrum has a major peak at 350 nm, a second peak at 368 nm, and a slight shoulder at about 330 nm. This compared well with the absorption spectrum, as would be expected. The small peak at 290 nm suggests there is energy transfer from tryptophan residues to the retinol. The emission spectrum peaks at 470 nm. Under the same conditions of buffer and pH, the spectra of rat CRBP are identical to those shown here for human CRBP.

Conclusion. The availability of an efficient method to purify human CRBP will allow extensive study of its properties to aid in understanding its role in the action of retinol in normal as well as neoplastic tissue. It should allow the comparison of CRBP from normal and cancerous tissue. Attempts are now underway to raise antibodies to this preparation which may lead to a sensitive radioimmunoassay for human CRBP in normal tissue as well as the possibility of immunolocalization of CRBP. It is presumed that the CRBP of cancerous tissue will prove to be identical to that of normal tissue. Such antibodies would then be valuable in studies on malignant tissue.

ACKNOWLEDGMENTS

The skillful technical assistance of Connie Turvy is gratefully acknowledged.

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