The binding protein for retinoic acid from rat testis cytosol: isolation and partial characterization

A. Catharine Ross, Noriaki Adachi, and DeWitt S. Goodman

Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032

Abstract This study reports the isolation and partial characterization of a soluble protein with binding specificity for retinoic acid from rat testis cytosol. Cytosol, labeled in vitro by incubation with [^{14}C]retinoic acid, was fractionated by a series of procedures that included ion exchange chromatography on DEAE-Sepharose and on DEAE-cellulose, gel filtration on Sephadex G-50, and preparative polyacrylamide gel electrophoresis. The resulting cytosol binding protein for retinoic acid had been purified approximately 16,000-fold, with recovery of 11% of the specifically bound [^{14}C]retinoic acid. The purified binding protein was homogeneous on disc gel electrophoresis at pH 4.5 or in the presence of SDS, but displayed microheterogeneity on isoelectric focusing, where both a major and a minor band with apparent isoelectric points near 4.7 were observed. Purified retinoic acid binding protein had a molecular weight of approximately 14,600, and an ultraviolet absorption spectrum with two maxima near 277 and 346 nm. The fluorescence intensity of retinoic acid bound to the cytosol binding protein was approximately 30-times greater than that of retinoic acid in solution in any of five organic solvents, suggesting that retinoic acid is highly immobilized when bound to the cytosol binding protein. The properties of the rat testis cytosol binding protein for retinoic acid were compared directly with those of the testis cytosol binding protein for retinol and of serum retinol-binding protein. The two cytosol binding proteins were much more similar to each other than to the serum transport protein, from which they differed in a number of properties, including molecular size, affinity for prealbumin, immunological reactivity, and absorption and fluorescence spectral characteristics.—**Ross, A. C., N. Adachi, and D. S. Goodman.** The binding protein for retinoic acid from rat testis cytosol: isolation and partial characterization. *J. Lipid Res.* 1980 **21:** 100–109.

Supplementary key words vitamin A · retinol · retinol-binding protein

Retinoic acid is a compound that demonstrates selective vitamin A biological activity. It is well established that retinoic acid is able to support a normal rate of body growth (1), as well as normal differentiation of epithelial tissues. Retinoic acid cannot, however, replace retinol as a visual pigment precursor (1), and is not able to support spermatogenesis in the male mammal (2), or normal fetal development in the female (3, 4). Thus, animals maintained on retinoic acid as the only source of vitamin A activity are both blind and sterile, but are otherwise in a good state of general health.

The role of retinoic acid in cellular differentiation is not yet known. Recently, however, intracellular binding proteins with specificity for either retinol or retinoic acid have been described in several tissues of the rat (5–7), man (7, 8), and other species (9–11). Certain analogs or isomers of retinoic acid have been found able to compete with all-*trans*-retinoic acid (7, 12, 13). Some of these analogs can regulate epithelial differentiation or display anticarcinogenic activity (14–16), and it has been reported that the binding ability tends to correlate with biological activity for given compounds (7, 13, 17). The possibility has thus been raised that the intracellular binding protein may mediate retinoic acid metabolism or function in target cells.

Recently, we and others have reported the purification of the intracellular binding protein for retinol, CRBP, from the cytosol of rat testis (18), rat liver (19), and bovine retina (20). Very recent reports on the purification of the intracellular binding protein for retinoic acid, CRABP, have also appeared (20–22). In our publication on the purification and properties of rat testis CRBP (18), information was also reported on the partial purification and on some of the properties of testis CRABP. We now report the complete purification of CRABP from rat testis cytosol, and describe spectral and other characteristics of the purified protein, as compared with those of purified CRBP and of serum RBP.

MATERIALS AND METHODS

Labeled compounds

All-*trans*-[15-^{14}C]retinoic acid (59 μCi/mg) was generously provided by Dr. W. E. Scott of Hoffmann-

Abbreviations: CRABP, cytosol binding protein for retinoic acid; CRBP, cytosol binding protein for retinol; RBP, serum retinol-binding protein; SDS, sodium dodecyl sulfate.

LaRoche, Inc., Nutley, NJ. Before use, the labeled compound was tested for purity by the solvent partition method of Borgström (23). More than 96% of the ^{14}C radioactivity partitioned as expected for a long chain fatty acid, and less than 1.7% partitioned as a nonacidic lipid. Unlabeled retinol and [11,12-$^{3}H_2$]-retinyl acetate were also provided by Hoffmann-LaRoche, Inc. [^{3}H]retinol was prepared (final sp act of 72 μCi/mg) by saponification of the [^{3}H]retinyl acetate and chromatography as previously described (18).

Preparation of cytosol

Cytosol was prepared by ultracentrifugation (142,000 *g* for 90 min) of homogenates from batches of 600 adult rat testes, and was labeled by the addition of [^{14}C]retinoic acid and [^{3}H]retinol to final concentrations of 1 nmol/ml of each compound exactly as described previously (18).

Column chromatography

All steps were conducted at about 5°C and in dim light. The chromatographic procedures used for purification of CRABP were essentially the same as those described in detail earlier for the purification of rat testis CRBP (18) except that an additional step (step 3, ion-exchange chromatography on DEAE-cellulose) was included to separate CRBP and CRABP from other non-retinoid-binding proteins prior to preparative electrophoresis. Details of steps 1, 2, 4, and 5 have been reported (18) and are stated here only in brief.

Steps 1 and 2: Ion-exchange chromatography of labeled cytosol on DEAE-Sepharose, pH 8.4, and gel filtration on Sephadex G-50 were performed as described (18). During DEAE-Sepharose chromatography, a single peak of material containing both [^{14}C]retinoic acid-labeled CRABP and [^{3}H]retinol-labeled CRBP was eluted at a conductivity of approximately 4 to 5 mS. After concentration by ultrafiltration (Amicon PM-10 Diaflo membrane) this pool of labeled material was applied to a large column (5.6 l) of Sephadex G-50 (fine) in buffer of 0.04 M Tris–HCl, 0.025 M NaCl, 0.002 M EDTA, 0.012 M thioglycerol, 0.02% Na azide, pH 8.4. Nearly coincident peaks of protein-bound ^{3}H and ^{14}C were eluted at a V_e:V_0 ratio of 1.5. The fractions comprising these peaks were combined into a single pool, which was concentrated and dialyzed (Spectrapor #1 dialysis membrane) against 0.01 M Tris–HCl buffer, pH 8.4, containing 0.001% Na azide, in preparation for ion-exchange chromatography on DEAE-cellulose.

Step 3: Whatman (DE52) DEAE-cellulose was equilibrated with buffer (0.1 M Tris–HCl, 0.01% Na azide) until the conductivity was less than 1.5 mS. The dialyzed protein sample, above, was applied to the column and eluted as described in the legend to Fig. 1.

Steps 4 and 5: Preparative polyacrylamide gel electrophoresis was performed exactly as reported previously (18). The separated pools of [^{14}C]retinoic acid-labeled CRABP and [^{3}H]retinol-labeled CRBP obtained from DEAE-cellulose chromatography were individually dialyzed, lyophilized, dissolved in a small amount of upper buffer [Davis buffer F (24)] containing 5% sucrose, and applied directly to the polyacrylamide resolving gel. After electrophoresis, fractions containing [^{14}C]retinoic acid were pooled, dialyzed against distilled water, and lyophilized.

Prior to recording spectra and conducting protein assays, purified CRABP was dissolved in 1 ml of buffer (0.05 M Na_2HPO_4, 0.02% Na azide, pH 8.0), and applied to a small column (1.3 × 50 cm) of Sephadex G-75 (superfine) equilibrated with the same buffer. This column separated a single peak containing all of the recovered [^{14}C]retinoic acid and part of the material that absorbed light at 280 nm from a later peak that contained only unlabeled, lower molecular weight material. This latter material also absorbed light at 280 nm but it did not stain with Coomassie Brilliant Blue dye. This second peak probably contained fragments of polyacrylamide that had eluted from the preparative electrophoresis gel along with CRABP.

Analytical methods

Polyacrylamide disc-gel electrophoresis was conducted at pH 8.9 according to Davis (24) and at pH 4.5 according to Potts et al. (25). SDS-disc-gel electrophoresis (26) was conducted as described earlier for CRBP (18). Analytical isoelectric focusing was performed in 6% polyacrylamide gel over a pH range of 4 to 6; gels were stained according to Reisner, Nemes, and Bucholtz (27). Absorption spectra, fluorescence spectra, and protein analyses were obtained or conducted as described previously (18).

Molecular weight estimation

The molecular weight of CRABP was estimated by SDS-polyacrylamide disc gel electrophoresis using 11% polyacrylamide gel, pH 7.1 (26). Standard proteins included bovine serum albumin, ovalbumin, chymotrypsinogen A, trypsin, myoglobin, cytochrome C, hemoglobin, lysozyme, and ribonuclease A. The molecular weight of CRABP was also estimated by gel filtration using a column of Sephadex G-75 and most of the protein standards above, as previously described (18).

Amino acid analysis

The partial amino acid compositions of rat testis CRABP and CRBP from the same cytosol preparation were determined after hydrolysis of 10 μg of protein in 6 N HCl for 24 hr at 110°C. The composition of human serum RBP (28) was also determined. Analyses were performed (29) by Drs. William Garner and Steven Birkin of this medical school, using a Beckman 121 MB amino acid analyzer.

Radioimmunoassay

CRABP, 1 to 1000 ng protein, was assayed in the radioimmunoassay for rat serum retinol-binding protein (30), as described previously for CRBP (18).

Extraction of ^{14}C-labeled ligand from CRABP

After final purification of CRABP, the ligand associated with 1.4 μg of CRABP was extracted in duplicate into ethanol. Using the solvent partition method of Borgström (23), ^{14}C radioactivity was partitioned into neutral and acidic lipid fractions and ^{14}C activity was assayed in each fraction.

Studies of retinoic acid and retinol in solution

All-*trans*-retinoic acid (Sigma) was dissolved under N_2 in redistilled ethanol containing butylated hydroxytoluene, 100 μg/ml. A solution of all-*trans*-retinol (Hoffmann-LaRoche, Inc.) was prepared similarly. Each stock solution was tested for purity by the solvent partition procedure (23). For retinoic acid, no absorbing material (A_{355} nm) partitioned into hexane from alkaline 50% ethanol and all of the absorbing material behaved as an acidic lipid. For retinol, 100% of the material absorbing light at 326 nm partitioned as a neutral (non-acidic) lipid and was recovered in the hexane phase after partition between hexane and alkaline aqueous ethanol.

For absorbance studies, aliquots of the stock solution of retinoic acid or retinol were dried under N_2 and immediately dissolved in redistilled ethanol, or spectral grade of methanol, hexane, cyclohexane, or benzene (Burdick and Jackson Laboratories). The absorption spectra of retinol or retinoic acid were recorded on a Beckman DB spectrophotometer equipped with a Photovolt recorder and using the appropriate solvent in the reference cuvette. Retinol in hexane with its known absorption maximum at 326 nm (31) was used to standardize the spectrophotometric data from experiment to experiment.

Fluorescence measurements and uncorrected fluorescence emission and excitation spectra were recorded on an Aminco-Bowman spectrophotofluorometer. Measurements were made on the same samples prepared for absorption spectroscopy which were subsequently diluted, so that response was proportional to concentration of retinoid for each solvent studied. Spectra were recorded at room temperature using light of the same wavelength as the absorption maximum to excite the fluorophore for fluorescence emission spectra. The maximum wavelength of fluorescence emission was used to record fluorescence excitation spectra.

RESULTS

Purification of CRABP

Purified CRABP was obtained from rat testis cytosol by a sequence of five steps which included *1*) ion-exchange chromatography on DEAE-Sepharose, *2*) gel filtration on Sephadex G-50, *3*) ion-exchange chromatography on DEAE-cellulose, *4*) preparative polyacrylamide gel electrophoresis, and *5*) gel filtration using Sephadex G-75. Simultaneously, rat testis CRBP was obtained in pure form, as described earlier (18). Although our previous purification scheme had been satisfactory for the isolation of pure rat testis CRBP, we had found that fractions containing rat testis CRABP (purified more than 340-fold) also contained a major contaminant which migrated together with CRABP during preparative gel electrophoresis (18). The purification sequence reported here included an additional chromatographic step, ion-exchange chromatography on DEAE-cellulose (**Fig. 1**), which efficiently separated [^{14}C]retinoic acid-labeled CRABP from the contaminant that lacked binding activity. In addition, use of DEAE-cellulose afforded some separation of CRABP from CRBP prior to preparative gel electrophoresis. Protein eluted in Pool 1 (Fig. 1) contained no radioactivity, and presumably comprised proteins other than CRBP and CRABP. On polyacrylamide gel, pH 8.9, protein from Pool 1 migrated very slightly cathodically to the [^{14}C]retinoic acid-labeled protein (partially purified CRABP, Pool 2). Pool 3 (Fig. 1) contained significant amounts of both CRABP and CRBP and was rechromatographed on DEAE-cellulose. The partially purified binding protein for retinol, CRBP, was eluted in Pool 4.

CRABP obtained after chromatography on DEAE-cellulose was estimated to have been purified more than 5000-fold. This material was then subjected to preparative polyacrylamide gel electrophoresis, and finally to gel filtration on Sephadex G-75. For the entire sequence of procedures, the apparent purification of CRABP was estimated as 16,000-fold (**Table 1**),

with a recovery of 11.4% of the specifically bound [^{14}C]retinoic acid from the labeled cytosol.

Analytical gels

Fig. 2 shows the final purified CRABP preparation as evaluated for homogeneity in four electrophoretic systems. CRABP was homogeneous in polyacrylamide gel (without SDS) at pH 4.5 (gel A), and by SDS-polyacrylamide disc-gel electrophoresis (gel B). Microheterogeneity of CRABP was observed after isoelectric focusing in gel (gel C) as well as in the alkaline system of Davis (24). In the latter two systems, one major band composed approximately 90% of the labeled protein and a second, minor and more anionic, band was also observed. The major band of CRABP clearly fluoresced when observed under ultraviolet light.

Although rat testis CRABP and CRBP were very nearly identical in size and chromatographic properties, they were distinguishable by different migration patterns on alkaline polyacrylamide gel and by isoelectric focusing: CRBP was clearly more anionic when electrophoresed in gel at pH 8.9 (gel E) than was CRABP (gel D). On isoelectric focusing, radioactivity associated with purified CRABP consistently focused at pH 4.6 to 4.7, whereas CRBP had an apparent isoelectric point of 4.8 to 4.9 (18).

Molecular weight

As estimated by SDS-disc gel electrophoresis, the molecular weights of pure CRABP and CRBP were

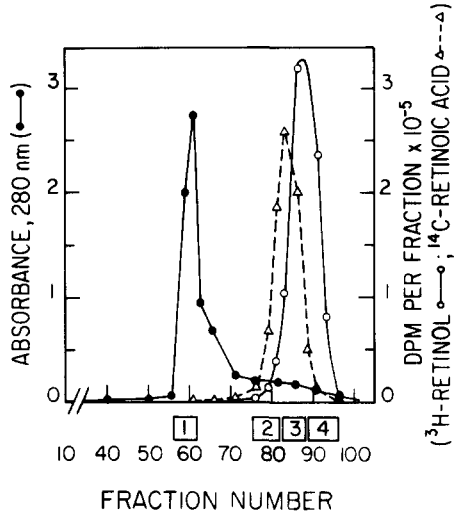

Fig. 1. Chromatography on DEAE-cellulose of partially purified CRABP and CRBP. The pooled protein obtained by gel filtration on Sephadex G-50 was dialyzed (see Methods) and applied to a 1.5 × 42 cm column of DEAE-cellulose in buffer (0.01 M Tris–HCl, 0.001% Na azide, pH 8.4). Elution was conducted at a flow rate of approximately 31 ml per hr with 200 ml of the same buffer, after which a linear gradient of NaCl, 0 to 0.2 M, was begun (total buffer volume approximately 700 ml). Fraction size was 5 ml. The major protein peak (A_{280} nm) eluted at a conductivity of approximately 4 mS; this protein peak contained neither ^{14}C nor ^{3}H radioactivity. Protein-bound [^{14}C]retinoic acid eluted at a conductivity of about 7 mS and [^{3}H]retinol bound to CRBP eluted last at a conductivity of about 9 mS. Fractions were combined into pools as indicated by the numbered bars along the abscissa. The pools containing both ^{3}H and ^{14}C radioactivity, or protein-bound [^{14}C]retinoic acid plus other unlabeled proteins (i.e., Pools 2 and 3), were rechromatographed on identical columns of DEAE-cellulose.

TABLE 1. Isolation of CRABP from rat testis cytosol

Procedure	Total A_{280}[a]	Recovery of Total A_{280}	Recovery of Specifically-bound [^{14}C]Retinoic Acid[b]	Apparent Specific Radioactivity[c]	Apparent Purification[d]
		%	%		
Labeled cytosol	5.92×10^{4}	(100)		9.03×10^{1}[e]	1.0
DEAE-Sepharose	2.98×10^{3}	5.03	100	1.79×10^{3}	2.0×10^{1}
Sephadex G-50	1.02×10^{2}	1.73×10^{-1}	73.1	3.82×10^{4}	4.2×10^{2}
DEAE-cellulose	7.27	9.02×10^{-3}	62.0	4.54×10^{5}	5.0×10^{3}
Preparative gel electrophoresis	1.41	2.38×10^{-5}	17.3	6.52×10^{5}	7.2×10^{3}
Sephadex G-75	2.10×10^{-1}	3.56×10^{-6}	11.4	1.47×10^{6}	1.6×10^{4}

[a] Total A_{280} = volume (in milliliters) × absorbance at 280 nm (A_{280}).

[b] Specifically bound [^{14}C]retinoic acid was estimated as 9.4% of the total [^{14}C]retinoic acid added to the cytosol. This estimate was obtained by assuming that all of the [^{14}C]retinoic acid recovered in the second labeled peak from the DEAE Sepharose column represented specifically bound [^{14}C]retinoic acid. This latter peak was previously shown (18) to contain the specific cytosol binding proteins for retinol and retinoic acid which had first been separated from other proteins on Sephadex G-50. In the preliminary studies reported previously (18), less than 9% of added [^{14}C]retinoic acid was eluted in the second (specifically-bound) peak from a Sephadex G-50 column. It is likely that the value used here (9.4% of added ^{14}C) somewhat overestimates the true amount (and recovery) of specifically bound [^{14}C]retinoic acid. Thus, the apparent purification listed (1.6×10^{4}) is probably a minimal estimate.

[c] Calculated as total disintegrations per min of specifically bound ^{14}C divided by total absorbance at 280 nm (i.e., disintegrations per min per unit of A_{280}).

[d] Expressed in cumulative terms.

[e] The apparent specific radioactivity of the labeled cytosol was calculated from the specifically bound [^{14}C]retinoic acid in the cytosol and the total A_{280}.

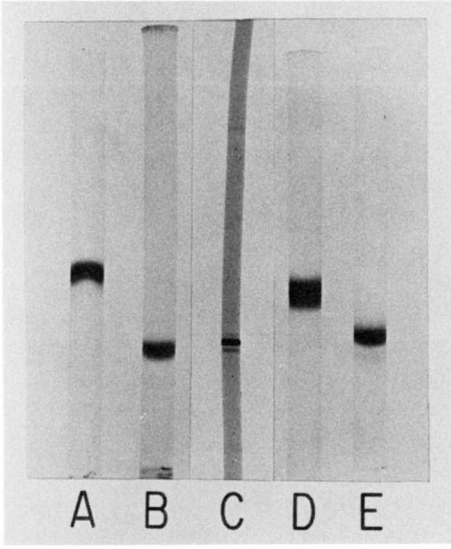

Fig. 2. Analyses of purified rat testis cytosol CRABP (gels A–D) and CRBP (gel E) by analytical electrophoresis (gels A, B, D, E) and by isoelectric focusing (gel C) in polyacrylamide gels. Gel A: CRABP (8 μg), 9% gel, pH 4.5; Gel B: CRABP (5 μg), SDS-polyacrylamide gel (11%, pH 7.1); Gel C: CRABP (10 μg) after isoelectric focusing in 6% gel using ampholytes of pH 4 (bottom) to pH 6 (top); Gel D: CRABP (10 μg) at pH 8.9; Gel E: CRBP (7 μg) at pH 8.9.

identical (14,600), indicating approximately the same linear dimensions. However, during molecular weight estimation by gel filtration, we have observed that the peak of [^{14}C]retinoic acid bound to CRABP consistently eluted just before the peak of [^{3}H]retinol bound to CRBP. This suggests that the radius of hydrated CRABP is slightly greater than that of CRBP. Molecular weight estimates from gel filtration on Sephadex G-75 provided values of 16,600 for CRABP and 15,900 for CRBP (18).

Amino acid analysis

Amino acid compositions of purified rat testis CRABP and CRBP, and of human serum RBP, were determined in order to compare these three retinoid-binding proteins. Due to the small amount of purified CRABP available at this time, only hydrolyses in HCl were performed. Thus, estimates were not obtained for the content of half-cystine or of tryptophan residues in these proteins. The partial amino acid composition obtained for human serum RBP (data not shown) was almost identical with the compositions previously reported from this laboratory (18, 32). The composition of CRBP (**Table 2**) differed somewhat from that previously reported (18). Rat testis CRABP and CRBP were quite similar to each other in overall amino acid composition (Table 2); differences observed between these two proteins included the presence of a lesser content of asx, and a greater content of thr and of ala, in CRABP as compared to CRBP. Serum RBP was distinguishable from both cytosolic binding proteins by its higher content of aromatic amino acids (tyr and phe). Each of the three retinoid-binding proteins contained a large proportion (greater than 25 mol %) of acidic amino acids or their amides.

Immunological comparison of rat testis CRABP and rat serum retinol-binding protein

Possible similarities between the cytosolic binding protein for retinoic acid and rat serum RBP were explored by immunoassay. Testis CRABP failed to compete with serum RBP for binding to antibodies raised against purified rat serum RBP when tested in the radioimmunoassay procedure for rat serum RBP. Thus, by this immunologic criterion, these retinoid binding proteins are distinctly different from one another.

Absorption and fluorescence spectroscopy of CRABP

The absorption spectrum of purified CRABP (**Fig. 3**) displayed two peaks, with maxima at approximately 277 nm and at 345 to 348 nm, respectively. The absorption spectrum of CRABP was almost identical to that of purified CRBP (see (18)). Thus, for both cytosol proteins, the second peak of absorbance, representing protein-bound ligand, had its maximum at approximately 346 nm. The absorption spectra showed definite shoulders at approximately 330 nm and 365–370 nm. Neither retinoic acid nor

TABLE 2. Amino acid composition of rat testis CRABP and CRBP[a]

Amino Acid	CRABP	CRBP
	residues per 100 residues analyzed	
Asx	11.76	14.31
Thr	8.61	5.58
Ser	4.86	3.82
Glx	13.51	14.86
Pro	3.46	2.46
Gly	8.77	9.55
Ala	6.93	4.31
Val	8.50	9.02
Met	2.27	3.33
Ile	4.98	4.22
Leu	8.03	8.69
Tyr	2.34	2.04
Phe	4.65	4.60
Lys	5.56	7.31
His	1.62	2.21
Arg	4.18	3.65
	100.03	99.96

[a] The values listed are the means of duplicate (for CRABP) or of quadruplicate (for CRBP) determinations from the HCl hydrolysate.

retinol in solution in organic solvents displays this fine structure. The ligand to protein absorption peak height ratio (i.e., A_{346}; A_{280}) was 1.22 (Fig. 3) for a preparation of CRABP estimated to contain 0.69 molecule retinoic acid per molecule binding protein (see below). Absorption spectra of CRABP were recorded both at pH 8.0 in Na phosphate buffer (Fig. 3) and at pH 4.5, slightly below the isoelectric point of CRABP, in Na phosphate-Na citrate buffer. Although the negative charge on CRABP was decreased at pH 4.5, no difference in the absorption spectrum of the bound ligand was observed.

The uncorrected fluorescence spectra of testis CRABP are shown in **Fig. 4.** When CRABP was excited at 340 nm, a peak of emission was recorded with its maximum at approximately 470 nm. The fluorescence excitation spectrum of CRABP (emission at 470 nm) showed a maximum at 355–360 nm. Thus, the fluorescence emission and excitation spectra of purified CRABP were qualitatively nearly identical with those of CRBP (18). Comparison of the fluorescence intensity per unit of absorbance, however, clearly indicates that the relative fluorescence intensity of the ligand bound to CRABP is much lower than that of ligand bound to CRBP. For CRABP having an absorbance at 346 nm of 0.35, the relative fluorescence intensity was 164 units. For CRBP purified from the same batch of testes and having an absorbance at 346 nm of 0.20, the relative fluorescence intensity was 460 units. Thus, the ratio of fluorescence intensity to absorbance for CRABP was approximately one-fifth as great as that for ligand bound to CRBP (466 versus 2300).

Estimation of amount of ligand bound to CRABP

An attempt was made to estimate the amount of ligand bound to the preparation of purified CRABP. The mass of total retinoic acid ligand was estimated from the absorbance at 346 nm, assuming that all bound ligand was retinoic acid with a molar extinction coefficient of 45,000 (33). The mass of exogenous, ^{14}C-labeled retinoic acid associated with the purified CRABP was calculated from the protein-bound radioactivity and the known specific radioactivity of the added retinoic acid. The amount of purified CRABP was estimated by protein assay according to Lowry et al. (34), and the molecular weight (14,600). According to these calculations, the molar ratio of exogenous retinoic acid to CRABP was 0.43, whereas the total retinoic acid to CRABP molar ratio was 0.69. Thus, using these assumptions, the data suggest that the added radioactive ligand bound to CRABP was significantly diluted by endogenous ligand also bound to the protein. Our calculations suggest that some endogenous, unlabeled, retinoid ligand was associated with CRABP in the cell, and that labeling the cytosol in vitro with [^{14}C]retinoic acid did not completely displace all of the endogenous ligand. In this preparation of CRABP, on the order of one-third to two-fifths of the total bound ligand appeared to be of endogenous origin.

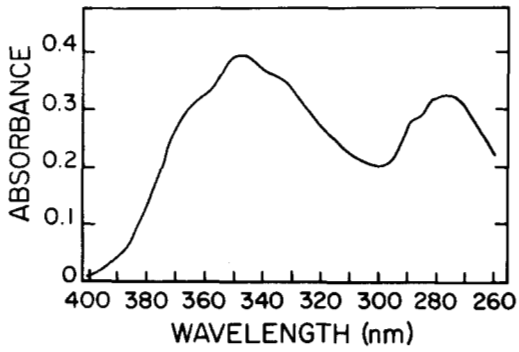

Fig. 3. Absorption spectrum of purified CRABP from rat testis cytosol, in solution in 0.05 M Na phosphate buffer, pH 8.0.

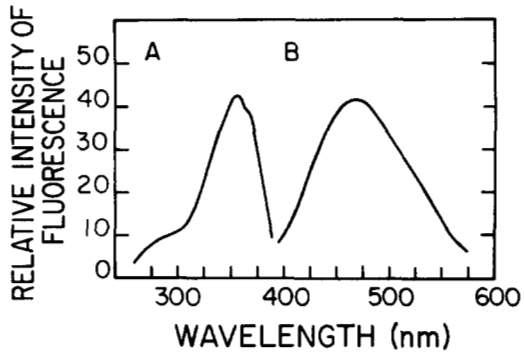

Fig. 4. Fluorescence excitation and emission spectra of purified CRABP at pH 8.0. A solution of CRABP having an absorbance at 346 nm of 0.352 was diluted 40-fold with Na phosphate buffer just before recording these spectra. A: Uncorrected excitation spectrum (emission at 470 nm); B: Uncorrected emission spectrum (excitation at 340 nm).

Extraction of ^{14}C-labeled ligand

In order to explore the question of whether the added, labeled retinoic acid was altered by its association with CRABP, we have extracted the ligand from purified CRABP and have determined its solvent partition characteristics. No ^{14}C radioactivity partitioned as a neutral lipid, indicating that none of the bound exogenous ligand had been reduced (e.g., to [^{14}C]retinol or retinal). After acidification of the aqueous ethanol phase to pH 1 and re-extraction with hexane, 97.4% of ^{14}C radioactivity that had been bound to CRABP was recovered in the hexane phase. Thus, the ^{14}C-labeled ligand behaved, in this system, as an acidic lipid, exactly as did the [^{14}C]retinoic acid that had been used to label the testis cytosol.

TABLE 3. Absorption and fluorescence characteristics of retinoic acid and retinol in organic solvents

Solvent	Retinoic Acid[a] Peak Maximum (nm) A	Retinoic Acid[a] Peak Maximum (nm) F[c]	Retinoic Acid[a] Relative Intensity of F to A[d] (Column 3)	Retinol[b] Peak Maximum (nm) A	Retinol[b] Peak Maximum (nm) F[c]	Retinol[b] Relative Intensity of F to A[d] (Column 6)	Ratio, Column 3 to Column 6[e]
Hexane	354–359	470–475	1.4	326	480	62	0.023
Cyclohexane	356–359	470–475	1.2	328	480	86	0.014
Benzene	363–365	470	1.2	330	480	95	0.013
Ethanol	340	470–480	1.7	326	480	40	0.043
Methanol	333	475	1.5	325	480	29	0.052

[a] Absorbance (A) and fluorescence emission (F) measurements were made using solutions of 7.7×10^{-6} M and 3.8×10^{-6} M retinoic acid, respectively. Values reported are the mean of two separate determinations.

[b] Absorbance and fluorescence measurements were made using solutions of 8.2×10^{-6} M and 1.6×10^{-6} M retinol, respectively. Values are the mean of two separate determinations.

[c] A standard solution of quinine sulfate in 0.01 N H_2SO_4 showed maximum fluorescence emission at 450 nm on this instrument.

[d] These columns list the relative intensity of fluorescence, per unit of absorbance, in arbitrary units. The same spectrophotofluorometer settings were used for all fluorescence readings reported in this paper so that all results are comparable.

[e] Calculated as relative intensity of F to A for retinoic acid divided by that for retinol.

Spectra of retinoic acid in organic solvents

The absorption and fluorescence spectra of pure retinoic acid in five organic solvents were examined. **Table 3** lists the position and relative heights of the absorption and fluorescence peak maxima for retinoic acid in each solvent. For comparison, retinol was examined at the same time in the same solvents. The absorption spectrum of retinoic acid was sensitive to solvent composition and to changes in acidity. In ethanol or methanol, the absorption spectrum of retinoic acid had its maximum value at 333–340 nm, but, upon addition of acid, the absorption peak maximum was shifted to approximately 355 nm (**Fig. 5**). This shift could be reversed by subsequent addition of base. The absorption spectra of retinoic acid in less polar solvents (hexane, cyclohexane, and benzene) resembled the spectrum of retinoic acid in acidified ethanol. In each solvent, the absorption spectrum appeared as a smooth curve, without shoulders.

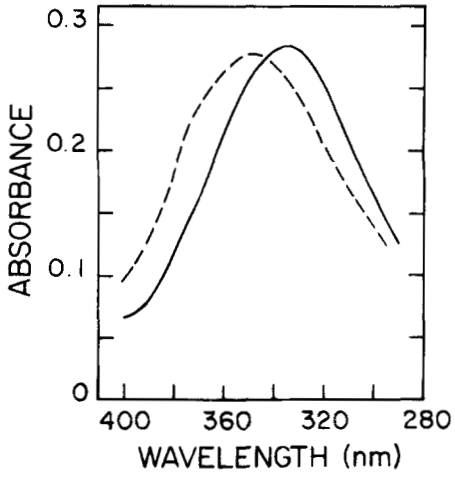

Fig. 5. Ultraviolet absorption spectra of retinoic acid in ethanol, and in acidified ethanol. Retinoic acid was dissolved in redistilled ethanol containing 100 μg butylated hydroxytoluene per ml solvent and the spectrum was recorded (——). The absorption spectrum was recorded again after addition of 10 μl of 0.01 N HCl (2.5×10^{-5} M H^+ final concentration) to the cuvette containing retinoic acid (– – –). The addition of 10 μl water in place of HCl had no effect on the absorption spectrum. Nearly identical spectra were observed when retinoic acid was dissolved in methanol instead of ethanol.

In order to quantitate and compare the fluorescence intensity of retinoic acid as a function of solvent composition, the relative fluorescence intensity per unit of absorbance was determined for each of the five solvents. The fluorescence emission of retinoic acid per unit of absorbance was greatest in ethanol and least in cyclohexane or benzene (Table 3, column 3). The variation of fluorescence emission intensity (per unit of absorbance) for retinol was much greater (>3-fold) than that of retinoic acid (1.4-fold). Comparing retinol to retinoic acid in each solvent (Table 3, column 7), the fluorescence yield per unit of absorbance for retinoic acid was always much less than that for retinol. Thus, in the different solvents, the relative intensity of fluorescence of retinoic acid (per unit of absorbance) varied from 1.4 to 5.2 percent of that of retinol in the same solvent.

The relative intensity of fluorescence (per unit of absorbance) of the retinoid ligand bound to CRABP was much greater than that of retinoic acid itself in solution in organic solvents. Using the same spectrophotofluorometer settings as those used for the data shown in Table 3, the relative intensity of fluorescence emission (470 nm) per unit of absorbance (at 346 nm) for CRABP was calculated to be 47. For retinoic acid

alone (Table 3), the corresponding values ranged from 1.2 to 1.7 for the five solvents tested. Thus, if the retinoid ligand bound to CRABP was entirely retinoic acid, its fluorescence emission intensity was enhanced 28- to 39-fold by binding to the protein.

DISCUSSION

This report describes the isolation and partial characterization of the binding protein for retinoic acid from rat testis cytosol. The CRABP had been purified at least 16,000-fold, with a yield of approximately 11%. CRABP both resembles, and is separate and distinct from, CRBP isolated from the same source. Within this past year, cellular binding proteins for retinoic acid have been purified in four laboratories from a variety of tissues. CRABP has been isolated from rat testis homogenates by both Ong and Chytil (21) and by ourselves. Very similar properties of the two purified preparations, including their molecular weights and absorption spectra, have been observed by the two laboratories. The present studies thus both confirm the observations of Ong and Chytil (21), and also provide additional information on the properties of CRABP, particularly as compared directly to rat testis CRBP and to serum RBP. CRABP has also been purified from bovine retina cytosol by Saari, Futterman, and Bredberg (20), and from chick embryo skin extracts by Sani and Banerjee (22). Despite these differences in starting materials, as well as differences in methods of purification, the major properties of the CRABP preparations obtained from these different sources were quite similar to each other. Whether these similar proteins from different species and tissues are also functionally homologous awaits further study. CRABP from rat testis has a molecular weight of 14,600 as estimated on SDS-disc gels, is anionic (pI 4.6 to 4.7), and contains a large proportion of acidic amino acids. Each molecule of binding protein appears to have a maximal binding capacity for one molecule of ligand (21).

The ultraviolet absorption spectrum of purified CRABP was characterized by two peaks with maxima at approximately 277 and 346 nm, and was almost identical with that previously observed with purified CRBP (18–20). From the relative heights of the two peaks we have estimated (see Results and ref. (18)) that both purified CRABP and purified CRBP from rat testis contain endogenous retinoid ligand, in addition to the radiolabeled retinoic acid or retinol added to the cytosol before purification. For the purified CRABP preparation reported here, we have estimated that the binding protein was approximately two-thirds saturated with respect to total retinoid (ligand to protein molar ratio of 0.69), with about 60% of the total bound ligand being comprised of the [^{14}C]retinoic acid that had been added in vitro.

The ^{14}C-labeled ligand extracted from purified CRABP partitioned as an acidic lipid, and thus retained the ionic character of retinoic acid. The exact chemical identity of the bound endogenous ligand is, however, not known. In this regard, the fine structure of the absorption spectrum of CRABP (and of CRBP (18–20)) is of interest. Definite shoulders at approximately 335 nm and 360 nm were observed on the second (retinoid) peak of the absorption spectrum of CRABP; similar fine structure was not observed for the absorption spectrum of retinoic acid alone in solution in organic solvents. It is possible that this fine structure reflects configurational effects resulting from the high affinity interaction of all-*trans*-retinoic acid and CRABP. Alternatively, it is possible that the endogenous ligand bound to CRABP in the cell is a derivative of all-*trans*-retinoic acid, with a somewhat different absorption spectrum from that of retinoic acid itself. For example, the absorption spectrum of 5,6-epoxyretinoic acid, a recently identified metabolite of retinoic acid (35), was observed to display fine structure in the form of shoulders on the main peak of absorbance (35). At the present time, further studies of the characteristics of the retinoid ligand extracted from pure CRABP have not been performed in this laboratory, because of the very limited amount of purified CRABP available. Further studies are needed, and would be of considerable interest, to establish the chemical identity of the endogenous ligand bound to CRABP in vivo.

The roles of the cytosol binding proteins for retinol and for retinoic acid in the testis of the rat remain to be defined. It is known that retinoic acid alone does not support mammalian spermatogenesis, and that retinol is required for maintenance of the testicular germinal epithelium (3, 36). The relationships between these observations and the intracellular binding proteins warrant exploration. In addition, since the testis consists of several different types of cells, the question of the cellular localization of the two different retinoid binding proteins needs to be examined.

A major focus of this laboratory for the past decade has been the study of the specific plasma vitamin A transport protein, RBP. RBP has been characterized extensively since its original isolation from human (28) and from rat (32) serum, with regard to a wide range of physical and chemical properties, and with regard to its physiological roles and its metabolic regulation (37, 38). Complementary studies on the transport of retinoic acid have shown that this ligand

is able to associate with RBP in vitro (31), but that the biological transport protein for retinoic acid in blood is serum albumin (39). Thus, the delivery of retinol and of retinoic acid to target tissues occurs by way of different transport systems.

With the purification of the intracellular binding proteins for retinol and for retinoic acid, it is now possible to compare the properties of each of the cytosolic binding proteins, CRABP and CRBP, to those of serum RBP. Both CRABP and CRBP from rat testis have a lower molecular weight (approximately 14,600) than rat serum RBP (approximately 20,000 (32)), and each has an amino acid composition that differs somewhat from that of serum RBP. By immunological criteria, CRABP and CRBP from rat testis cytosol are each clearly different from serum RBP, since neither protein showed any immunological cross-reactivity with rat serum RBP when examined in the radioimmunoassay for RBP. Whether the two cytosolic binding proteins for retinoids are also immunologically distinct from one another remains to be determined. Unlike serum RBP, neither testis CRBP nor CRABP showed affinity for serum prealbumin bound to Sepharose (18). In all of these properties the two cytosol binding proteins resembled each other, but differed from serum RBP.

Distinct differences between serum RBP and the intracellular retinoid binding proteins were also found when spectral characteristics were examined. The binding of retinol to serum RBP results in a blue shift of about 15 nm in the fluorescence emission maximum, and in an approximately 10-fold enhancement of fluorescence emission intensity of the retinol (40). These data suggest that retinol is bound tightly to serum RBP in a relatively fixed state (40). In contrast, rat testis CRBP displayed a fluorescence emission maximum nearly identical to that of retinol in solution in ethanol (18), and a much smaller enhancement of fluorescence emission intensity. Thus, the fluorescence intensity for the retinoid ligand bound to CRBP was found to be only one-fourth that of retinol bound to serum RBP (18). These observations suggest that retinol is not so rigidly immobilized when associated with CRBP, as compared to serum RBP.

Only limited information is available on the fluorescence characteristics of retinoic acid. Thomson (41) could detect fluorescence of retinoic acid in hydrocarbon solvent only at very low temperature, whereas others (42) have reported that retinoic acid does fluoresce, but very weakly when compared to retinol or to retinyl esters. As reported here, the yield of fluorescence from retinoic acid was clearly detectable in each of five different solvents, but was very low (0.014 to 0.052) relative to the fluorescence of retinol, of equivalent absorbance, in each of the solvents studied.

Comparison of the relative intensity of fluorescence emission of CRABP with that of retinoic acid in solution in organic solvents or with that of CRBP, suggests that the fluorescence intensity of retinoic acid is greatly enhanced when bound to CRABP. Saari et al. (20) have also recently reported that the intensity of fluorescence emission of retinoic acid is enhanced upon binding to bovine retinal CRABP. If we assume that the retinoid ligand bound to CRABP was entirely retinoic acid, then our data indicate that its fluorescence emission intensity was enhanced approximately 30- to 35-fold by binding to the protein. As a result, the relative intensity of fluorescence of CRABP (per unit of absorbance) was approximately one-fifth as great as that of CRBP. These data suggest that retinoic acid is quite highly immobilized when bound to CRABP, more similar to the case of retinol bound to serum RBP than to that of retinol bound to CRBP.

We extend our appreciation to Dr. John E. Smith for valuable advice and discussion, and for the immunoassay of CRABP, and to Drs. W. Garner, A. Spector, S. Birkin, and R. Canfield for amino acid analyses. We thank Mr. R. Brown for expert technical assistance, and Dr. W. E. Scott, of Hoffmann-La Roche, Inc., for the gift of [15-^{14}C]-retinoic acid. Dr. Ross's present address is: Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Room 318-R, 3300 Henry Avenue, Philadelphia, PA 19129. This research was supported by Grant AM 05968 from the National Institutes of Health.

Manuscript received 4 May 1979; accepted 13 September 1979.

REFERENCES

1. Dowling, J. E., and G. Wald. 1960. The biological function of vitamin A acid. *Proc. Nat. Acad. Sci. (USA)* **46:** 587–608.
2. Thompson, J. N., J. McC. Howell, and G. A. J. Pitt. 1964. Vitamin A and reproduction in rats. *Proc. Roy. Soc. London.* Ser. B. **159:** 510–535.
3. Howell, J. McC., J. N. Thompson, and G. A. J. Pitt. 1964. Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. II. The female rat. *J. Reprod. Fertil.* **7:** 251–258.
4. Takahashi, Y. I., J. E. Smith, M. Winick, and D. S. Goodman. 1975. Vitamin A deficiency and fetal growth and development in the rat. *J. Nutr.* **105:** 1299–1310.
5. Bashor, M. M., D. O. Toft, and F. Chytil. 1973. In vitro binding of retinol to rat-tissue components. *Proc. Nat. Acad. Sci. (USA)* **70:** 3483–3487.
6. Ong, D. E., and F. Chytil. 1975. Retinoic acid binding protein in rat tissue. Partial purification and comparison to rat tissue retinol-binding protein. *J. Biol. Chem.* **250:** 6113–6117.
7. Chytil, F., and D. E. Ong. 1976. Mediation of retinoic

acid-induced growth and anti-tumour activity. *Nature.* **260:** 49–51.
8. Swanson, D., S. Futterman, and J. C. Saari. 1976. Retinol- and retinoic acid-binding proteins: occurrence in human retina and absence from human cultured fibroblasts. *Invest. Opthalmol.* **15:** 1017–1020.
9. Sani, B. P., and T. H. Corbett. 1977. Retinoic acid-binding protein in normal tissues and experimental tumors. *Cancer Res.* **37:** 209–213.
10. Saari, J. C., and S. Futterman. 1976. Separable binding proteins for retinoic acid and retinol in bovine retina. *Biochim. Biophys. Acta.* **444:** 789–793.
11. Wiggert, B. O., D. R. Bergsma, and G. J. Chader. 1976. Retinol receptors of the retina and pigment epithelium: further characterization and species variation. *Exp. Eye Res.* **22:** 411–418.
12. Sani, B. P., B. C. Titus, and C. K. Banerjee. 1978. Determination of binding affinities of retinoids to retinoic acid-binding protein and serum albumin. *Biochem. J.* **171:** 711–717.
13. Sani, B. P., and D. L. Hill. 1976. A retinoic acid-binding protein from chick embryo skin. *Cancer Res.* **36:** 409–413.
14. Chopra, D. P., and L. J. Wilkoff. 1977. Reversal by vitamin A analogues (retinoids) of hyperplasia induced by N-methyl-N′-nitro-N-nitrosoguanidine in mouse prostate organ cultures. *J. Natl. Cancer Inst.* **58:** 923–939.
15. Sporn, M. B., N. M. Dunlop, D. L. Newton, and W. R. Henderson. 1976. Relationships between structure and activity of retinoids. *Nature.* **263:** 110–113.
16. Sporn, M. B., N. M. Dunlop, D. L. Newton, and J. M. Smith. 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proc.* **35:** 1332–1338.
17. Jetten, A. N., and M. E. R. Jetten. 1979. Possible role of retinoic acid binding protein in retinoid stimulation of embryonal carcinoma cell differentiation. *Nature.* **278:** 180–182.
18. Ross, A. C., Y. I. Takahashi, and D. S. Goodman. 1978. The binding protein for retinol from rat testis cytosol. Isolation and partial characterization. *J. Biol. Chem.* **253:** 6591–6598.
19. Ong, D. E., and F. Chytil. 1978. Cellular retinol-binding protein from rat liver. Purification and characterization. *J. Biol. Chem.* **253:** 828–832.
20. Saari, J. C., S. Futterman, and L. Bredberg. 1978. Cellular retinol- and retinoic acid-binding proteins of bovine retina. Purification and properties. *J. Biol. Chem.* **253:** 6432–6436.
21. Ong, D. E., and F. Chytil. 1978. Cellular retinoic acid-binding protein from rat testis. Purification and characterization. *J. Biol. Chem.* **253:** 4551–4554.
22. Sani, B. P., and C. K. Banerjee. 1978. Purification and properties of retinoic acid-binding protein from chick-embryo skin. *Biochem. J.* **173:** 643–649.
23. Borgström, B. 1952. Investigation on lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. *Acta Physiol. Scand.* **25:** 111–119.
24. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121:** 404–427.
25. Potts, J. T., Jr., R. A. Reisfeld, P. F. Hirsch, A. B. Wasthed, E. F. Voelkel, and P. L. Munson. 1967. Purification of porcine thyrocalcitonin. *Proc. Nat. Acad. Sci. (USA)* **58:** 328–335.
26. Weber, K., and M. Osborn. 1975. Proteins and sodium dodecyl sulfate: molecular weight determination on polyacrylamide gels and related procedures. *In* The Proteins. H. Neurath, and R. L. Hill, editors. Academic Press, New York. 3rd Ed., Vol. 1. 179–223.
27. Reisner, A. H., P. Nemes, and C. Bucholtz. 1975. The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Anal. Biochem.* **64:** 509–516.
28. Kanai, M., A. Raz, and D. S. Goodman. 1968. Retinol binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47:** 2025–2044.
29. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30:** 1190–1206.
30. Smith, J. E., Y. Muto, and D. S. Goodman. 1975. Tissue distribution and subcellular localization of retinol-binding protein in normal and vitamin A-deficient rats. *J. Lipid Res.* **16:** 318–323.
31. Goodman, D. S., and A. Raz. 1972. Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein. *J. Lipid Res.* **13:** 338–347.
32. Muto, Y., and D. S. Goodman. 1972. Vitamin A transport in rat plasma. Isolation and characterization of retinol-binding protein. *J. Biol. Chem.* **247:** 2533–2541.
33. Planta, C. V., U. Schweiter, L. Chopard-dit-Jean, R. Rüegg, M. Kofler, and O. Isler. 1962. Synthesen in der Vitamin-A_2-Reihe. Physikalische Eigenschaften von isomeren Vitamin-A- und Vitamin-A_2-Verbindungen. *Helv. Chim. Acta.* **45:** 548–561.
34. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
35. McCormick, A. M., J. L. Napoli, H. K. Schnoes, and H. F. DeLuca. 1978. Isolation and identification of 5,6-epoxyretinoic acid: a biologically active metabolite of retinoic acid. *Biochemistry.* **17:** 4085–4090.
36. Ahluwalia, B., and J. G. Bieri. 1971. Local stimulatory effect of vitamin A on spermatogenesis in the rat. *J. Nutr.* **101:** 141–152.
37. Goodman, D. S. 1976. Retinol binding protein, prealbumin, and vitamin A transport. *In* Trace Components of Plasma: Isolation and Clinical Significance G. A. Jamieson, and T. J. Greenwalt, editors. Alan R. Liss, Inc., 313–330.
38. Smith, J. E., and D. S. Goodman. 1979. Retinol-binding protein and the regulation of vitamin A transport. *Federation Proc.* **38:** 2504–2509.
39. Smith, J. E., P. O. Milch, Y. Muto, and D. S. Goodman. 1973. The plasma transport and metabolism of retinoic acid in the rat. *Biochem. J.* **132:** 821–827.
40. Goodman, D. S., and R. B. Leslie. 1972. Fluorescence studies of human plasma retinol-binding protein and of the retinol-binding protein-prealbumin complex. *Biochim. Biophys. Acta.* **260:** 670–678.
41. Thomson, A. J. 1969. Fluorescence spectra of some retinyl polyenes. *J. Chem. Phys.* **51:** 4106–4116.
42. Thompson, J. N., P. Erdody, R. Brien, and T. K. Murray. 1971. Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* **5:** 67–89.