Interphotoreceptor retinol-binding protein (IRBP) is a unique retinol carrier: it is a large glycoprotein existing only in the interphotoreceptor matrix (IPM), which is the extracellular material situated between and behind the photoreceptors of the neural retina. IRBP from bovine and human eyes carries endogenous retinol, as evidenced by the protein's fluorescence on gel-filtration (Sephacryl S-300) chromatography and on native (non-SDS) pore-gradient polyacrylamide gel electrophoresis. Bovine IRBP's retinol-binding sites are at most one-third occupied in light-adapted eyes and much less in dark-adapted eyes; this bleach dependence is partially reversible and suggests a role in vitamin A transport during the visual cycle. IRBP can be saturated with exogenous all-trans retionol; one ligand molecule binds per protein molecule. IRBP can be isolated rapidly from IPM by affinity adsorption onto con A-Sepharose; this preparation is 94% pure and yields 0.33 mg or 2.4 nmol of IRBP per bovine eye. An apparently homogeneous preparation can be obtained by subsequent passage through a Sepharose 4B column. IRBP is located only in the IPM, which is harvested by isotonic washing of the retina. (In the absence of this rinse to prepare IPM, the protein is found loosely associated with the retina, although not as a cellular or membrane component.) As the thoroughness of the retinal washing procedure is increased, the yield of IPM proteins (including IRBP) goes up; however, the same set of proteins, in the same ratios, always is retrieved, indicating that retinal-cell components do not contaminate seriously the IPM.

The sequence of vitamin A transformations during the visual cycle has been known for some time: retinaldehyde, released from rhodopsin by bleaching, is converted to retinol in the retina, somehow transported to the subjacent retinal pigment epithelium (RPE), and esterified and stored there. These processes are reversed in the dark, with vitamin A conserved. The mechanism of concomitant retinol transport is not yet understood. One possibility would be by direct passage across adjacent plasma membranes of photoreceptor outer segments and of the RPE cells in which they are partially ensheathed; however, retinol is toxic to cell membranes.

Probably as a consequence of this toxicity and of retinol's insolubility, in living organisms vitamin A appears always to be linked, noncovalently, to some binding protein. These proteins include serum retinol-binding protein in the blood and cellular retinol-binding protein in the cytosol of many epithelial tissues, including neural retina and RPE. In addition, there are two vitamin-A carriers unique to the eye and, therefore, probably involved in specific visual functions. These are cellular retinaldehyde-binding protein in the retina and RPE and interphotoreceptor retinol-binding protein (IRBP) in the interphotoreceptor matrix (IPM). (This large retinol-carrying protein was detected first in association with the retina.)

The IPM is the thin layer of protein-rich extracellular material that occupies the subretinal space in the normal vertebrate eye. Since photoreceptor outer segments and RPE apical microvilli project into the IPM, this space is placed strategically to function in transport between retina and RPE. Because of its location, and because the amount of retinol it binds is controlled by the eye's state of light adaptation, IRBP is a likely candidate for a physiologic visual-cycle retinol carrier.

We took advantage of the fact that IRBP is the major glycoprotein in the IPM to isolate the protein rapidly, with some contamination, from bovine IPM by affinity adsorption onto con A-Sepharose. We have now purified IRBP to apparent homogeneity. In this report we show that IRBP is confined to the extracellular subretinal space and begin to examine its retinol-binding properties.
Materials and Methods

Preparations of Interphotoreceptor Matrix (IPM)

Adult bovine eyes were obtained from local meat packers, human eyes from the Massachusetts Eye and Ear Infirmary eye bank. Eyes were transported to the laboratory in the dark. Unless other conditions are given, eyes were light-adapted (LA) as in Adler and Martin,12 and procedures were carried out at 4°C. For dark-adapted (DA) IPM preparations, DA eyes were dissected under dim red light (GE type BAS red light bulb plus Kodak 1A filter). The operational definition of IPM was taken to be the soluble material lying in the subretinal space (between the retina and the RPE), material that can be collected by rinsing the formerly apposed surfaces of these two tissues following separation. IPM was isolated by our modifications16 of the Berman and Bach method,19 in which IPM is isotonically washed from these tissues.

In the usual IPM preparative procedure for this study (the 45-min stir method, which optimizes IRBP yield), 10–30 bovine eyes were trimmed to remove connective tissue and then were cut just posterior to the ora serrata. The anterior portion was discarded. The vitreous was removed carefully, without causing retinal or choroidal detachment. Final traces of vitreous were removed by washing the anterior retinal surface with phosphate-buffered saline (PBS), consisting of 0.14 M NaCl + 5 mM sodium phosphate, pH 7.4. Retinas were excised by cutting at the optic nerve. They were pooled in PBS (2 ml/eye), stirred at the slowest speed (20 rev/min) on a magnetic stirrer for 30 min and then filtered through plastic mesh. The filtrate was saved, and the extraction yield), 10–30 bovine eyes were trimmed to remove connective tissue and then were cut just posterior to the ora serrata. The anterior portion was discarded. The vitreous was removed carefully, without causing retinal or choroidal detachment. Final traces of vitreous were removed by washing the anterior retinal surface with phosphate-buffered saline (PBS), consisting of 0.14 M NaCl + 5 mM sodium phosphate, pH 7.4. Retinas were excised by cutting at the optic nerve. They were pooled in PBS (2 ml/eye), stirred at the slowest speed (20 rev/min) on a magnetic stirrer for 30 min and then filtered through plastic mesh. The filtrate was saved, and the extraction procedure was repeated for 15 min (utilizing 1 ml buffer/eye) on the residual retinas. The two retinal filtrates (washes) were combined. Meanwhile, material buffer/eye) on the residual retinas. The two retinal or choroidal detachment. Final traces of vitreous were removed by washing the anterior retinal surface with phosphate-buffered saline (PBS), consisting of 0.14 M NaCl + 5 mM sodium phosphate, pH 7.4. Retinas were excised by cutting at the optic nerve. They were pooled in PBS (2 ml/eye), stirred at the slowest speed (20 rev/min) on a magnetic stirrer for 30 min and then filtered through plastic mesh. The filtrate was saved, and the extraction procedure was repeated for 15 min (utilizing 1 ml buffer/eye) on the residual retinas. The two retinal filtrates (washes) were combined. Meanwhile, material

Other Tissue Preparations

Retinas were either excised from the eyecup and left unwashed or were used after removal of IPM by the various rinsing methods stated above. RPE cells were isolated by brushing the eyecup20; the supernatant from the first centrifugation step was processed along with the cells to avoid loss of cytoplasm.21 Tissue cytosols were obtained by homogenizing these tissues in twice their own volume of PBS, and retaining the supernatants after centrifugation (20,000 × g for 80 min) of the homogenates. The resulting residues comprised the membrane fractions.

Purification of Interphotoreceptor Retinol-binding Protein (IRBP)

Since IRBP is the major glycoprotein of the IPM,17 the first step used in isolation was affinity adsorption onto concanavalin A-Sepharose.18 Briefly, IPM was stirred with con A-Sepharose beads in buffer. The beads were washed. The “con A fraction” (or nearly pure IRBP) then was liberated by stirring the beads with α-methyl-D-mannoside. The beads were removed by filtration, and the protein was concentrated by passage through a macrosolute concentrator (Amicon B-15 Minicon).

After con A treatment the preparation contained about 6% impurity (as seen by PAGE). This was removed by passage through a Sepharose Cl-4B column (0.9 × 30 cm) cluted with PBS and monitored by protein absorbance at 280 nm. Fractions (1 ml each) of the principal peak, displaying at least half-maximum absorbance, were pooled. This preparation consisted of essentially homogeneous IRBP.

Analyses

Approximate protein concentrations of IPM preparations and cytosols were determined by the method of Lowry et al.22 Concentrations of purified IRBP were calculated from the absorbance at 280 nm; the extinction coefficient based on weight (E280, 1% = 8.7) was measured by dialyzing ~2-mg samples of IRBP exhaustively against water, drying to constant weight over P2O5, and weighing on a Cahn electrobalance.

The retinol content of IPM preparations was assayed fluorimetrically after extraction into petroleum ether.23 This work was carried out in dim red light; samples were kept under nitrogen. Fluorescence intensity was measured with a Perkin Elmer MPF-44A
fluorescence spectrophotometer at excitation wavelength 330 nm, emission 480 nm.

Treatment with Exogenous Retinol

Some samples of IPM and IRBP were loaded with all-trans-retinol (Eastman) prior to chromatography or electrophoresis: retinol in a small amount of ethanol was allowed to incubate with the sample for 30 min at room temperature in the dark. When saturation of IRBP binding sites was desired, a fivefold molar excess of retinol was used. See figure legends for specific experiments.

Gel-Filtration Chromatography

Samples of IPM and cytosols were passed through a Sephacryl S-300 column to fractionate protein components according to size. Since the major goal was to detect retinol-binding proteins, fractions were monitored by retinol fluorescence. (See Figure 1 for details.)

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis of various preparations was performed on acrylamide-gradient slab gels, in the presence and absence of sodium dodecyl sulfate (SDS), using modifications of O’Farrell’s methods. Linear gradients and stacking gels were used. (Procedures are given in Figures 3 and 4.) Most gels were stained for protein with Coomassie blue; silver staining also was used in some cases. Negatives from photography of these gels were scanned with a Joyce-Loebl densitometer; areas of peaks were measured with a Hewlett-Packard digitizer. Some native (non-SDS) gels were photographed in such a way that bound-retinol fluorescence was recorded (see Figure 5).

Results

Retinol-binding proteins in the interphotoreceptor matrix: Effect of Light Adaptation

When bovine IPM, prepared by our usual method, is passed through an analytic gel-filtration column monitored by retinol fluorescence (Fig. 1), two peaks are observed. The larger protein is interphotoreceptor retinol-binding protein (IRBP); the smaller is cellular retinol-binding protein (CRBP). The “250 K” marker serves only to indicate IRBP’s apparent size in relation to protein standards and to identify it in the elution pattern. (Molecular-sieving methods, including both this type of column and pore-gradient electrophoresis [see later], overestimate the molecular weight [M<sub>r</sub>] of the asymmetric [but not dimeric] IRBP molecule; its real M<sub>r</sub> is 133,000 ± 2000, as measured by sedimentation equilibrium.

Much more endogenous retinol is carried by IRBP in the light-adapted (LA) eye than in the dark-adapted (DA). (This effect was mentioned but not documented in Adler and Martin and also was noted by Liou et al.) Since the amount of IRBP actually present in the eye is invariant with respect to light-dark adaptation (see later), bleaching apparently causes more retinol to bind to IRBP. The same result for LA eyes (3.5 ± 0.2 fluorescence units per eye) is obtained whether light adaptation is achieved by brief exposure to bright light (2 min at 15 cm from a 150-watt white bulb) or by 1–3 hr under ambient indoor illumination. When whole LA eyes subsequently are dark-adapted (for 1.5 hr), this effect is largely (75%) reversible; the endogenous retinol on IRBP decreases to 1.3 fluorescence units per eye, comparable to the 0.6 ± 0.1 units bound to IRBP in eyes initially dark adapted. When this cycle is carried one step farther, and the eyes then are exposed to light again, 2.5 fluorescence units of endogenous retinol are bound. When an excess of exogenous all-trans-retinol is added to either LA- or DA-IPM, the binding sites on IRBP are saturated, and the same level of fluorescence is observed for both. The small amount of CRBP present in the IPM...
Fraction number

Fig. 2. Gel-filtration chromatography of human IPM from light-adapted eyes. Same conditions as for Figure 1. Endogenous fluorescence was monitored.

is always retinol-saturated, in keeping with its behavior in retina and RPE. 11

Figure 1 displays gel-filtration data for bovine IPM prepared in the winter (November–March); LA-IRBP is approximately 30% saturated with endogenous retinol. In summer (June–October), LA-IRBP in the IPM binds only 1.4 fluorescence units per eye, or about 10% of saturation. 12 This difference has been consistent for two annual cycles and may somehow reflect seasonal variations in housing and/or diet of the animals (winter: indoor illumination, fed with hay; summer: outdoor, fresh grass; Johnston Dressed Beef and Veal Co.; Johnston, RI, personal communication).

IRBP is present also in the human eye (Fig. 2). The smaller size of the human protein ("210 K") is in agreement with its lower Mr, found by SDS-PAGE (human: 131 K; bovine: 140 K). The peak at "80 K" is probably the serum retinol-binding protein–prealbumin complex. (Human, but not bovine, IPM contains several serum proteins; Adler and Martin, in preparation.) We have used SDS-PAGE to identify IRBP also in rat, rabbit, and frog IPM; the protein is missing in chicken. Both the bovine 18 and the human IRBP molecules are stripped of their ligand during isolation by the con A-affinity procedure; both can be recharged to saturation by incubation with exogenous all-trans-retinol and yield fluorescent peaks at the appropriate positions upon Sephacryl chromatography.

Purity and Yield of Bovine IRBP

SDS-PAGE patterns at various stages of IRBP preparation are given in Figure 3. Lanes h, k, and l
indicate our usual procedure, in which the retina is rinsed isotonically and fairly vigorously until the yield of IRBP is constant. Bovine IPM (lane h) contains over 20 proteins. IRBP is seen at 140 K; 47 K refers to another major IPM band. Con A treatment results in a preparation consisting of 94% IRBP, as measured by scanning of gel densitometric traces (Table 1), and a contaminating band at 55 K. This impurity can be removed by passage through a Sepharose column; lane l appears virtually homogeneous. (There is <1% impurity, with no other band above the noise level in scans; this is confirmed by silver staining, as in the densitometric scan to the right of lane l. If such a freshly prepared sample is stored several days at 4°C or −20°C, a high Mₐ, diffuse band begins to build up, probably indicative of aggregation.)

Gel electrophoresis of undenatured proteins (in the absence of SDS) is another method for probing the composition of samples. Pore-gradient gels, in which electrophoresed native proteins reach equilibrium positions dependent primarily upon their size, are shown in Figure 4. IRBP runs at an apparent Mₐ of 225,000, just under catalase (at 232 K). (As with the related method of gel-filtration chromatography, this Mₐ value is artifically high; “225 K” merely serves as a marker to designate an apparent molecular weight determined using standard proteins.) The initial IPM preparation can be approximated by lane c, since IPM-P (lane d) contributes only about 8% of the total IPM protein (Table 2). Lane g shows a fresh preparation of IRBP, apparently homogeneous. After prolonged storage, a small band at ~500 K (perhaps dimer) sometimes appears. One advantage of native PAGE is that binding proteins retain their ligands. The fluorescence of these gels is displayed in Figure 5, which will later be examined in the “retinol binding” section.

Gel-filtration chromatography alone is insufficient as a preparative method for IRBP. Only 27% of the appropriate Sephacryl-column peak consists of IRBP (Fig. 3, lane j; Fig. 4, lane e; Table 1). An additional pass through Sepharose removes only part of the contaminating proteins.

The amount of protein recovered after con A treatment (0.33 ± 0.03 mg per eye; average from eight samples) is 5% of the total IPM protein yield (Table 1, column 2). (About 60% of this then is discarded in the Sepharose step.) This 0.33 mg total protein includes 0.31 mg IRBP per bovine eye. Since the molecular weight is 133,000, this yield corresponds to 2.4 ± 0.3 nmol per eye. The same amount of IRBP was isolated from DA as from LA preparations. The molar extinction coefficient of IRBP at 280 nm, 11.6 × 10⁴ M⁻¹ cm⁻¹, does not change significantly.

Table 1. Purification of IRBP: recovery and retinol binding

<table>
<thead>
<tr>
<th>Preparative step</th>
<th>Total protein recovered (mg/eye)</th>
<th>Total IRBP</th>
<th>Absorbance ratio, A₃₈₀/A₂₈₀</th>
<th>Molar ratio, retinol/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPM</td>
<td>6.6 ± 0.1</td>
<td>0.12</td>
<td>0.06</td>
<td>0.15</td>
</tr>
<tr>
<td>Sephacryl peak*</td>
<td>1.4 ± 0.1</td>
<td>0.07</td>
<td>0.13</td>
<td>0.30</td>
</tr>
<tr>
<td>Con A affinity</td>
<td>0.33 ± 0.01</td>
<td>0.04</td>
<td>0.39</td>
<td>0.93</td>
</tr>
<tr>
<td>Con A plus</td>
<td>0.19 ± 0.01</td>
<td>0.01</td>
<td>0.43</td>
<td>1.03</td>
</tr>
<tr>
<td>Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pooled fractions 65-73 from Sephacryl S-300 column; see Figure 1.
† Assayed by Lowry method.
‡ Measured by absorbance at 280 nm. All protein determinations have average errors of ±10% for triplicate preparations.
§ Ratio of 140 K band area to that of total protein, obtained from scans of SDS gels.
¶ Spectra run after saturation with exogenous all-trans-retinol and corrected for contribution of free retinol.
|| Calculated from data of previous column, using extinction coefficients for retinol and IRBP.

Fig. 4. Native, gradient PAGE in the absence of SDS. Usual IPM preparations, and stages in purification of IRBP. (See Fig. 3 legend for more complete description of samples.) Lane a, high-Mₐ standards; lane b, low-Mₐ standards; lane c, IPM-R, prepared by 45-min stir; lane d, IPM-P; lane e, “250-K” peak from Sephacryl chromatography of IPM; lane f, IPM after con A treatment; lane g, IRBP (IPM after both con A and Sepharose steps). Samples were run on 5~18% linear-gradient PAGE slabs in tris-glycine buffer. Gels were stained with Coomassie blue and photographed. The calibration plot (log of apparent Mₐ versus migration distance) was linear only for Mₐ > 30 K.
 Constancy of IPM-R Prepared by Various Methods

We chose a rather vigorous preparative method for IPM to maximize the yield of protein (and of IRBP). Other investigators\(^{13,28}\) washed the retina more gently to avoid any possible contamination from damaged retinal or RPE cells, even though SDS gels of Adler and Severin\(^{16}\) indicated that this is not a serious problem. We, therefore, compared IPM-R (which contains the bulk of IPM protein) obtained by several different isotonic-rinsing methods.

Figure 3 (lanes c–e) shows that the same set of proteins, in the same proportions, is always obtained. (The state of light adaptation also causes no change, lane f; neither does the season.) Table 2 shows, specifically, that the percent of IRBP in IPM-R is invariant (11 ± 1%), and that the amount of retinol (in LA preparations), as well as of IRBP, is always proportional to the total amount of protein. The major protein at 47 K on SDS-PAGE always comprises 9 ± 1%. Therefore, the protein composition of IPM-R is constant; extraneous components from surrounding tissues do not progressively contaminate this material. Thus a preparative procedure designed to maximize yield is justified. Note that (Table 2) the yield from the IPM-R preparation of Liou et al\(^{13}\) is about 40% of ours, by all criteria used. The retinol content of DA preparations is 20–30% that of LA preparations.

In comparison to IPM-R, the IPM washed from the RPE surface (IPM-P), although containing the same set of proteins, is enriched greatly in IRBP (Fig. 4, lane d; Table 2). This corroborates the immunocytochemical finding of Bunt-Milam and Saari\(^{29}\) that IRBP is most concentrated in the IPM at the RPE apical surface. When IPM is prepared by lavage of the subretinal space (“no-cut” method), the preparation is low in yield and looks remarkably similar in composition to IPM-P. It seems likely that the saline introduced cannot adequately penetrate between photoreceptor outer segments unless the retina is detached fully from the RPE. (A recent method\(^{30}\) for extremely gentle lavage of monkey subretinal space results in an IPM preparation in which IRBP is the only major protein.)

IRBP is Localized to the IPM

The next sets of experiments were designed to distinguish whether IRBP is truly an extracellular protein, present only in the interphotoreceptor matrix, or whether it is, at least in part, a component of retinal-cell cytoplasm or membranes. Such a distinction is important in elucidating IRBP’s role in vision. When the retina is washed only briefly, some IRBP is present in the retinal homogenate supernatant (cytosol).

Figure 6 presents endogenous retinol-fluorescence patterns for retina cytosol from retinas either not washed at all (that is, excised and homogenized with IPM-R still clinging to their surface) or thoroughly...
2. Compositional consistency of different interphotoreceptor matrix preparations

Table 2. Compositional consistency of different interphotoreceptor matrix preparations

<table>
<thead>
<tr>
<th>Source of IPM</th>
<th>Washing method</th>
<th>Total protein (mg)</th>
<th>IRBP (mg)</th>
<th>Retinol (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina</td>
<td>10-sec dip</td>
<td>0.6</td>
<td>0.02</td>
<td>0.12</td>
</tr>
<tr>
<td>Retina</td>
<td>15-min soak*</td>
<td>2.6</td>
<td>0.11</td>
<td>0.32</td>
</tr>
<tr>
<td>Retina</td>
<td>5-min stir</td>
<td>2.9</td>
<td>0.10</td>
<td>0.41</td>
</tr>
<tr>
<td>Retina</td>
<td>15-min stir</td>
<td>4.7</td>
<td>0.16</td>
<td>0.58</td>
</tr>
<tr>
<td>Retina</td>
<td>45-min stir†</td>
<td>6.1</td>
<td>0.25</td>
<td>0.92</td>
</tr>
<tr>
<td>RPE</td>
<td>rinse‡</td>
<td>0.5</td>
<td>0.08</td>
<td>0.23</td>
</tr>
<tr>
<td>Interface</td>
<td>no-cut‡</td>
<td>1.2</td>
<td>0.11</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* Method of Liou et al., 1982.
† Assayed by Lowry method.
‡ Isolated by Con A affinity method; concentration measured by absorbance at 280 nm.
|| Ratio of 140 K band area to that of total protein, obtained from scans of SDS gels.

Retinol Binding to IRBP

As seen in Figure 1, most of the retinol-binding sites of IRBP are unoccupied even before the protein is isolated from the IPM. The con A step of purification removes nearly all of the endogenous retinol. The same report showed, through Sephacryl-chromatography data, that isolated IRBP, as well as IRBP in the IPM, can be recharged with exogenous retinol.

These observations are confirmed by variations that can be caused in the protein-bound retinol fluorescence displayed by IRBP on native, pore-gradient PAGE (Fig. 5). The fluorescence of IRBP ("225 K" band) in the crude interphotoreceptor matrix (lane a) is enhanced greatly by incubation with exogenous retinol (lane b). On the other hand, the faint fluorescence of CRBP ("<30 K") remains unchanged, in agreement with the data of Figure 1. (Although such small proteins are beyond the M_r calibration range of these gels, the identification of this band as CRBP is corroborated by its presence, fully saturated with retinol, in RPE cytosol, as shown in lanes g and h.) Lane c indicates the barely visible fluorescence of purified IRBP; lanes d–f show how its binding sites can be filled progressively with exogenous retinol. There is no IRBP in the RPE; this confirms findings from SDS gels and gel-filtration chromatography.

Saturating levels of retinol were added to the IPM preparations after each step of the IRBP purification procedure, and UV absorption spectra were run and corrected for the contribution of unbound retinol. A set of such curves has been presented for the preparation after the con A step. The retinol-to-protein ratios thus obtained (Table 1) are in good agreement with IRBP-to-total-protein ratios from SDS-PAGE. (CRBP contributes to A_330 of crude IPM, making it somewhat higher than expected.)

Purified IRBP holoprotein also was obtained and...
Fraction number

Fig. 6. Gel-filtration chromatography to demonstrate conservation of IRBP during preparation of IPM. All curves show endogenous retinol fluorescence of light-adapted preparations. Cytosol (containing soluble proteins) from unwashed retina, —; cytosol from retina that had been stirred in PBS for 45 min to remove IPM-R, — — — ; IPM-R resulting from this wash, ---. Same conditions as for Figure 1. Vₑ = excluded volume of column, as measured with blue dextran.

analyzed by a more accurate method: the IPM preparation, after the con A treatment stage, was incubated with retinol (10 nmol per eye), and then passed through a Sepharose column, which removed both excess retinol and contaminating traces of protein. The spectra from such samples were very similar to those corrected for free retinol. The protein molar extinction coefficient at 280 nm for chromatographed samples is the same, within experimental error, for the apoprotein ($11.4 \times 10^4$) and the holoprotein ($11.7 \times 10^4$). Furthermore, the molar extinction coefficient of all-trans retinol, $4.6 \times 10^4$ M$^{-1}$ cm$^{-1}$ in ethanol, is unchanged upon binding to IRBP, since $A_{330}$ of pure, saturated IRBP remains constant after extraction of retinol into petroleum ether. From these data we find the molar stoichiometry of retinol binding to be 1:1. (See also Table 1.)

Because of a very recent report$^{32}$ of a different stoichiometry, we reexamined our procedures in an attempt to load more retinol onto IRBP. These modifications included gentler IPM preparations (15-min soak and no-cut), addition of 1 mM dithiothreitol (to prevent possible disulfide formation) and of 0.1 mM phenylmethylsulfonyl fluoride (to prevent proteolysis), deletion of the concentrating step, and more rapid gel-filtration chromatography (HPLC with a TSK 4000 SW column or passage through a 2-cm column of Sepharose). In our hands, the binding ratio remained 1:1.

Discussion

Bovine interphotoreceptor retinol-binding protein has been purified, in a two-step procedure, to apparent homogeneity when examined by the criteria of gel-filtration chromatography, SDS-PAGE, native-protein pore-gradient PAGE (and sedimentation velocity in the analytical ultracentrifuge$^{26}$). It is now feasible to begin physicochemical studies on this protein. Since the other retinol-binding protein present in crude preparations of interphotoreceptor matrix, CRBP (probably a contaminant$^{30}$ from the RPE), has been removed, ligand-binding properties measured will be those of IRBP alone. The physiologic relevance of this protein is increased by the finding that it is present in human eyes.$^{12,33}$

We have shown that the composition of IPM preparations is constant and not dependent on how the retina is washed; progressive tissue contamination is not observed. This finding is justification for optimization of IPM protein yield in studies of IPM components including, but not limited to, IRBP.

Biochemical analysis (PAGE and fluorescence of column fractions) indicates that IRBP is an extracellular protein, located exclusively in the subretinal space. IRBP clings to the bordering tissue surfaces

Fig. 7. Densitometric tracings of SDS-PAGE gels. Absorbance is plotted against distance down the gel lane. SDS gels were run as in Figure 3. "IPM" shows a scan for the usual preparation, yielding a gel like lane h of Figure 3. The "retina cytosol" scans show the soluble proteins remaining with the retina after the various treatments noted for removal of IPM-R. "Retina membranes" indicates the combined residues (after centrifugation) from 45-min stir preparations of IPM-R and retina cytosol; the membranes were solubilized in 1% SDS. Arrows show position of IRBP. Several Mᵢ markers are given, to facilitate comparison with Figure 3.
when the retina is detached from the RPE. However, a simple rinse with isotonic buffer (to harvest the IPM) is sufficient to remove IRBP from these surfaces (including from outer segment plasma membranes and from RPE apical microvilli). IRBP is not a cellular retinol carrier; it is present neither in the cytoplasm nor in the membranes of retina or RPE. This biochemical evidence for IRBP localization to the IPM agrees with immunocytochemical findings and, furthermore, shows (as light microscopy cannot) that IRBP is not incorporated into the bordering cell membranes.

IRBP displays two properties essential for any physiologic retinol shuttle functioning in the visual cycle—it is situated in proximity both to the photoreceptor outer segments and to the RPE, and the amount of retinol it carries endogenously is a function of lighting conditions. More IRBP-bound retinol would be expected—and is found—following bleaching. (This is mainly all-trans-retinol, the major natural ligand of IRBP, possibly on its way to the RPE. Preliminary results show that the small residual load of retinol present on IRBP in the dark is enriched in the 11-cis isomer, perhaps returning slowly to the retina. We find the 11-cis:all-trans ratio for endogenous, IRBP-bound retinol to be 0.3 in the light and 1.2 in the dark.) Apparently, 2 min of bright light is sufficient to optimize the loading of vitamin A onto IRBP. Light-modulated changes in IRBP retinol binding are largely reversible as eyes are cycled twice from dark to light. This finding is additional evidence for a role for this protein in visual function; presumably, reversibility is complete in the living animal. The reason for the observed seasonal variation in IRBP retinol binding is unknown. (The CRBP found in the IPM is always fully saturated with retinol; because of this insensitivity to light adaptation, this binding protein is probably not involved in this transport phase of the visual cycle.)

We routinely isolate and assay (by absorbance) 2.4 nmol of IRBP per bovine eye after the con A affinity-adsorption step in purification. However, analysis of fluorescence chromatograms following saturation with exogenous retinol and passage through a Sephacryl column indicates that approximately 30% of the IRBP present in crude IPM has been lost by this time (most of its because of the concentrating procedure). Thus the amount of IRBP actually present in a bovine eye is probably 3–4 nmol. (This estimate agrees well with a recent immunoelectrophoretic assay.) The bovine eye contains 23 nmol of retinaldehyde (on rhodopsin) in the dark-adapted state. Therefore, IRBP has the capacity to carry about 15% of the vitamin A released during a complete bleach of the bovine retina. (An estimate of 20% has been given for the human eye.) This amount of retinol-binding capacity should be quite sufficient to remove the retinol released for nearly all lighting conditions encountered during life.

One question arises: if retinol is present in excess of IRBP in the light-adapted IPM, why is IRBP not saturated with endogenous ligand under conditions of complete bleaching? (30% of saturation is the highest observed, although calculations based on the dissociation constant predict >99% saturation.) Perhaps our experimental procedures are not optimal for obtaining an IPM preparation soon enough after retinol release. Alternatively, since much of the IRBP is localized near the RPE border, perhaps the protein cannot all be mobilized at the retina for shuttling at any one time. Another possibility is that much of the released vitamin A may remain associated with the photoreceptors.

Several links in the mechanism for visual-cycle vitamin A transport are, as yet, unexplored. One important problem is how retinol passes through outer segment- and RPE apical-plasma membranes. These steps are likely to involve a membrane-bound retinol-binding protein receptor. Although IRBP is not an intrinsic membrane protein, it is associated with these surfaces, and may bind to such a receptor.

Key words: interphotoreceptor matrix, retina, vitamin A, retinol-binding proteins, protein purification.

References
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