Mechanism of Vitamin A Movement between Rod Outer Segments, Interphotoreceptor Retinoid-binding Protein, and Liposomes

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Vitamin A movement between rod outer segment (ROS) membranes, interphotoreceptor retinoid-binding protein (IRBP), and liposomes was examined by two different methods. Equilibrium exchange of all-trans-retinol was followed by assessing the transfer of \[^{3}H\]retinol from liposomes to ROS membranes as compared to a nontransferable marker, \[^{14}C\]triolein. In the absence of IRBP, a rapid, spontaneous transfer of \[^{3}H\]retinol to the ROS membranes occurred. In the presence of \(2 \mu M\) IRBP, retinol transfer decreased by approximately one-half, whereas a similar concentration of bovine serum albumin had no effect on this spontaneous transfer. Kinetics of retinol transfer between single unilamellar vesicles were determined by the method of fluorescence energy transfer. The first order rate constant for this transfer was \(0.85 \text{ s}^{-1}\) at \(22^\circ\text{C}\) at either \(pH\) 7.4 or 2.8. This rate was not affected by varying the concentration of acceptor vesicles 50-fold or by varying their concentration 10-fold at a constant ratio of donor-to-acceptor vesicles. The presence of IRBP as an additional acceptor did not change the rate. The transfer was temperature-dependent with an activation energy of 7.8 kcal/mol. The transfer rate appeared to be an increasing exponential function of ionic strength since high concentrations of NaCl decreased the transfer rate significantly. The transfer rate of retinol from IRBP to single unilamellar vesicles also followed first order kinetics with a rate constant of \(0.11 \text{ s}^{-1}\) at \(22^\circ\text{C}\), which was approximately 8 times slower than that of transfer between vesicles. We conclude that the transfer of all-trans-retinol between liposomes and membranes can be accomplished rapidly \(via\) the aqueous phase, and that IRBP retards rather than facilitates this transfer process.

Transfer of retinoids between photoreceptor outer segments and the retinal pigment epithelium is an important step in the visual cycle that leads to the regeneration of rhodopsin.

IRBP\(^1\) is a high molecular weight glycolipoprotein that binds retinoids, fatty acids, and vitamin E, and is found exclusively in the extracellular space of the interphotoreceptor matrix and the pineal gland (1, 2). Because of its unique localization, it has been repeatedly suggested (1–4) that IRBP serves to transport retinoids between outer segments of the photoreceptor cells and the retinal pigment epithelium, i.e. to transport all-trans-retinol released from outer segments to the retinal pigment epithelium where isomerase (5) and retinyl ester synthetase (6) are localized, and to deliver 11-cis-retinoid back to the outer segments for rhodopsin regeneration. However, no evidence has been presented to demonstrate the direct involvement of IRBP in this shuttle function.

A large body of evidence has accumulated which indicates that short chain fatty acids, bilirubin, and estrone have fairly rapid spontaneous transfer between liposomes (7–10), with a half-time in the range of seconds, while long chain fatty acids, cholesterol, and vitamin E move between liposomes with a half-time in the range of minutes to hours (8, 11, 12). The transfer of pyrene-labeled fatty acids has been studied by monitoring the time-dependent excimer fluorescence changes following mixing of donor and acceptor vesicles (7). It was concluded that the transfer took place through the aqueous phase because it was a first order process and was independent of the concentration of acceptor membranes. The transfer rate was a function of both hydrophobicity and hydrophilicity of the transferred molecule, i.e. it increased both with decreased chain length and increased polarity of the molecule (8). Transfer of cholesterol between liposomes (13) and of vitamin E between lipoproteins (12) also followed first order kinetics, although the rates were much slower. The mechanism of transfer of bilirubin or estrone from liposomes to microsomes could not be ascertained because the methods applied did not provide a direct measurement of the transfer kinetics (10, 14).

The transfer of retinoids between liposomes has previously been examined by Rando and Bangert (15). They performed conventional transfer experiments and concluded that the rate of spontaneous transfer of free retinoids was too rapid to be determined by their method, but the half-time was estimated to be about 1–2 min. Fex and Johannesson (16) also applied similar methodology to examine the transfer of retinol from SRBP to unilamellar liposomes and concluded that the spontaneous transfer occurred with a half-time of about 30

\(^{1}\) The abbreviations used are: IRBP, interphotoreceptor retinoid-binding protein; SRBP, serum retinol-binding protein; CRBP, cellular retinol-binding protein; CRALBP, cellular retinaldehyde-binding protein; BSA, bovine serum albumin; ROS, rod outer segments; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PBS, phosphate-buffered saline; SUV, single unilamellar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmityl-1-PE.
min. However, the method used in both studies could not directly measure the rate of transfer, and the precise kinetics and mechanism of this transfer remains unknown.

The nature of the transfer of all-trans-retinol between liposomes and ROS, IRBP and ROS, and IRBP and liposomes using the methods of equilibrium exchange of \([^{3}H]\)retinol (compared to a nontransferable marker) and fluorescence energy transfer. The latter has previously been used to examine membrane fusion and intermembrane transfer of a variety of other hydrophobic molecules (12, 17, 18). We were particularly interested to know the route of transfer between membranes and the role of IRBP in this transfer. If retinoids are transferred via the aqueous phase, then IRBP may serve only as a buffer protein for retinoids in the interphotoreceptor matrix. If the transfer is via the lipid phase, then IRBP may provide an important transport molecule for retinoids between the photoreceptor cells and the retinal pigment epithelium, because a direct contact of the plasma membranes of these two cell types has not been documented, even though they are often in close proximity when observed with electron microscopy. Our studies demonstrate that the transfer of all-trans-retinol between single unilamellar vesicles takes place in the lipid phase. These findings suggest that IRBP may serve only as a buffer to allow gradual exchange of retinoids between membrane compartments that border the interphotoreceptor matrix.

**Experimental Procedures**

Materials—Egg PC, bovine brain PE, concanavalin A-Sepharose, and all-trans-retinol, [9,10-\(^{3}H\)]palmitic acid, and glycerol tri[1,4-C]oleate (triolein) were obtained from Amersham. Sepharose CL-4B and Sephadex G-100 were purchased from Pharmacia LKB Biotechnology Inc. N-[7-Nitrobenz-2-oxa-1,3-diazol-4-yl]dipalmitoyl-\(\alpha\)-PE (NBD-PE) was obtained from Molecular Probes (Eugene, OR). Frozen bovine retinas were purchased from Hormel (Rochester, MN).

**Purification of Bovine IRBP**—Preparation of the interphotoreceptor matrix and purification of IRBP was according to the method of Fong et al. (19) with the following modification. Bovine eyes were obtained from a local slaughterhouse and were processed within 3 h after they were enucleated. An equatorial cut was made along the ora serrata, and the anterior segment and vitreous were removed. The retinas were then carefully peeled away from the RPE surface and soaked for 5 min successively in each of three beakers containing 10 ml PBS (5 mM NaHPO4, 1.5 mM NaCl, pH 7.4) at 4°C. PBS (3 ml) was placed into each eyecup and left to soak for 5 min. The contents of the three beakers and the RPE rinses were combined and centrifuged at 100,000 x g for 1 h. The supernatant was applied to a column of concanavalin A-Sepharose. The concanavalin A-bound fraction was eluted with the same buffer containing 0.01 M NaCl and 200 mM methyl-D-mannopyranoside. The eluant was dialyzed against PBS overnight, and the volume was reduced with a microconcentrator (Centricon-30, Amicon, M, = 30,000 cut-off) to approximately 2 mg/ml protein. Approximately 3 ml of this solution was applied to a Sepadex G-100 column (2 x 100 cm) at ambient temperature, and the column was eluted with PBS. The first major peak was collected and consisted of more than 95% pure IRBP.

**Preparation of Single Unilamellar Vesicles**—Egg PC (20 mg) in chloroform with 0.1 mol % butylated hydroxytoluene was placed in a screw cap test tube wrapped with aluminum foil along with 8 mg of PC and dried by a stream of nitrogen. Residual chloroform was removed by mechanical vacuum. Tri-NaCl buffer was added to a final lipid concentration of 2 mg/ml. The suspension was added to a water bath (15-20°C) for 2 h. The dispersed lipid vesicles were then centrifuged at 100,000 x g for 30 min, and the supernatant was used for experiments after proper dilution with Tris/NaCl buffer (50 mM Tris-HCl, 1 mM EDTA, 0.14 M NaCl, pH 7.4). The final concentration of phospholipid in the vesicle suspension was determined by the method of Rouser et al. (20) and ranged from 0.5-5.0 μg of phosphorus/ml in the various preparations.

**Preparation of ROS Membranes**—Bovine ROS membranes were prepared according to the method of Lee et al. (21). Frozen bovine retinas were thawed in a cold room under dim red light. Each group of 25 retinas was placed in a 50-ml polyethylene centrifuge tube containing 30 ml of sucrose (density 1.13 g/ml), 5 mM Tris-HCl, 2 mM MgCl2, and 62 mM NaCl, pH 7.4. The tubes were shaken vigorously for 1 min. The supernatants were centri- fuged at 10,000 x g for 12 min. The supernatants were diluted with 2 volumes of 50 mM Tris-HCl and 5 mM MgCl2, pH 7.6, and centrifuged at 27,000 x g for 30 min. The resultant ROS pellets were washed three times with Tris/NaCl buffer and finally resuspended in the same buffer and stored at -70°C in the dark.

**Equilibrium Exchange Experiments**—Liposomes containing [\(^{3}H\)]all-trans-retinol or [\[^{3}H\]^palmitic acid were prepared as follows. [\(^{3}H\)]all-trans-Retinol in ethanol (10 μl, 60 Ci/mmol, 1 mCi/ml) or [\[^{3}H\]^palmitic acid in toluene (10 μl, 54 Ci/mmol, 1 mCi/ml) was added to 2 ml of liposomes (1.2 mC PC containing [\(^{3}H\)]triolein and sonicated for 10 min before each experiment. These liposomes (final phospholipid concentration, 0.1 mM) were incubated in a microcentrifuge tube with ROS (0.2-0.5 mM phospholipid) in Tris-NaCl buffer at a final volume of 1 ml. IRBP or BSA (2 μg) was present in some of the incubations. The incubations were begun by the addition of ROS and were terminated by centrifugation for 10 min. It took about 1 min to isolate the ROS membranes by the method of Rouser et al. (20) of the supernatant were collected at various times, and their \(^{3}H\)/\(^{14}C\) ratios were determined by scintillation spectrometry after mixing with 5 ml of aqueous counting scintillant (Amersham). A decrease in the \(^{3}H\)/\(^{14}C\) ratio in the supernatant indicated the transfer of all-trans-retinol to the ROS membranes. The results were compared to the counts from the samples obtained at the beginning of incubations. Recovery of [\(^{3}H\)]triolein in the supernatant was always more than 90%. The liposomes and IRBP were further separated by gel filtration chromatography. An aliquot of supernatant (0.75 ml) was subjected to gel filtration on Sepharose CL-4B (1.5 x 30 cm) at ambient temperature, and the column was eluted with Tris/NaCl buffer. Fractions of 0.75 ml were collected, and \(^{3}H\)/\(^{14}C\) ratios were determined in each fraction. The concentration of protein was determined by the method of BCA protein assay (Pierce) with BSA as the protein standard (22).

**Stopped-Flow Experiments**—NBD-PE (0.4 mg) in chloroform was placed in a screw cap test tube wrapped with aluminum foil along with 8 mg of PC and dried by a stream of nitrogen. Residual chloroform was removed by mechanical vacuum. Tri-NaCl buffer was added to a final lipid concentration of 2 mg/ml. The suspension was vortexed briefly and then placed in a bath sonicator for 1 h. The suspension was then centrifuged at 100,000 x g for 30 min, and the supernatant was used as donor vesicles. all-trans-Retinol in ethanol was added to donor vesicles to a concentration of 0.14 M (lipid K). The final concentration of all-trans-retinol (0.01 mol % PC) before each experiment, and the mixture was sonicated for 5 min. All solutions were degassed prior to use by mechanical vacuum for 20 min and then flushed with nitrogen for 10 min.

The instrumentation and methods used in the stopped-flow experiments have been described previously (23). The time-dependent fluorescence change was monitored by SLM mode 8000 digital single photon counting spectrofluorometer. Because NBD-PE absorbs moderately around 330-350 nm, we chose to excite at 355 nm where retinol in single unilamellar vesicles has 80% of its maximal absorption, but NBD-PE has negligible absorption. Fluorescence emission was measured with a 450-470 nm narrow band interference filter (Melles-Griot, Irvine, CA). All experiments were done in Tris/NaCl buffer unless mentioned otherwise. For retinol transfer between single unilamellar vesicles with or without IRBP, the initial retinol concentration in donor vesicles was 3-5 mol % PC. For transfer from IRBP to single unilamellar vesicles, retinol in 100% ethanol was added to IRBP in Tris/NaCl buffer at a molar ratio of 1:1 (final concentration of ethanol was 0.2 volume %), and the time-dependent fluorescence emission was monitored with a computer-driven SFA-11 rapid kinetics stopped-flow apparatus (HI-Tech Scientific Limited, Salisbury, England) was used. With this accessory placed within the cuvette holder of the fluorometer, 200-μl aliquots of the two reactants could be mixed in less than 20 ms. The data were stored in a Biomation Model 805 wave form recorder and subsequently transferred to an Apple II Plus computer using the Apple Quickdraw program to obtain a first order rate constant. In each experiment, the transfer rates presented are the average of at least three traces.

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RESULTS

Equilibrium Exchange Experiments

In agreement with the results obtained by Rando and Bangerter (15), we found a rapid spontaneous transfer of all-trans-retinol between PC liposomes and ROS membranes. Under the experimental conditions, about 40% of the all-trans-retinol initially present in liposomes was transferred to ROS membranes within 1 min when the donor-to-acceptor (ROS membranes) ratio was 1:5 (Fig. 1). However, in the presence of 2 μM IRBP, the amount of spontaneous transfer decreased to around 25%, although the transfer was also completed within 1 min. A similar concentration of BSA had no significant effect on the spontaneous transfer. However, if 20 μM BSA was used, a decrease in total transfer to 32%, or about half the effect of 2 μM IRBP, was observed. The recovery of IRBP or BSA was usually more than 90%, indicating that adsorption of IRBP or BSA to the ROS membranes was insignificant.

After separation of ROS membranes by centrifugation, a portion of the supernatant was subjected to a Sepharose CL-4B column, and two peaks were observed (Fig. 2). The first peak represented liposomes as judged by the co-migration of 3H and 14C in a fixed ratio. The second peak contained IRBP (judged by protein assay) and its bound ligand, [3H]retinol. Approximately 90% of the 14C was associated with liposomes, but 3H was distributed between liposomes and IRBP. The effect of IRBP on the spontaneous transfer was concentration-dependent, as shown in Fig. 3. IRBP at a concentration less than 0.25 μM had no observable effect on the spontaneous transfer. As the concentration of IRBP increased, the amount of transfer decreased. These findings led us to conclude that the effect of IRBP on spontaneous transfer was due to the binding of a portion of retinol to IRBP rather than the inhibition of the transfer process. Because of the time required to separate the ROS membranes by centrifugation, this procedure did not allow us to determine the kinetics of spontaneous transfer. Therefore, these data only reflect the equilibrium distribution of all-trans-retinol between liposomes, ROS membranes, and IRBP.

Likewise, the spontaneous transfer of palmitic acid was rapid and significant. Within a minute, almost 85% of palmitic acid initially present in the liposomes was transferred to the ROS membranes when the ratio of their concentration was 1:5. However, the effects of BSA and IRBP were the reverse of that observed for retinol transfer (Fig. 4). BSA at a concentration of 2 μM reduced by more than one-half the spontaneous transfer of palmitic acid (from 85% to 40%),

FIG. 1. Transfer of all-trans-retinol from liposomes to ROS membranes. SUV (0.1 mM PC) containing [3H]all-trans-retinol (about 1 mol%) and a trace amount of [14C]triolein (less than 0.1 mol%) were incubated with ROS membranes (0.5 mM phospholipid) at room temperature in Tris-EDTA buffer, pH 7.4, in the absence or presence of either 2 μM IRBP or 2 μM BSA. The transfer of [3H]retinol was determined as described under "Experimental Procedures." In the absence of IRBP or BSA, the equilibrium was reached within 1 min, and about 40% of the retinol initially present on the liposomes spontaneously transferred to ROS membranes. In the presence of 2 μM IRBP, the equilibrium was also reached within 1 min, but the amount of spontaneous transfer decreased to 25%. A similar concentration of BSA had only a slight effect on the spontaneous transfer. The calculated percentages of transfer had an experimental error of 3–4%.

FIG. 2. Separation of liposomes and IRBP by gel filtration. Experiments were conducted as described in Fig. 1. After separation of the ROS membranes by centrifugation, 0.75 ml of the supernatant (containing IRBP and liposomes) was placed on a Sepharose CL-4B column. Two peaks were observed. The first peak contained both 3H and 14C in a fixed ratio and represented liposomes, while the second peak contained only 3H and represented IRBP.

FIG. 3. The effect of concentration of IRBP on the spontaneous transfer of all-trans-retinol. At a concentration of 0.25 μM, IRBP had no observable effect on the spontaneous transfer. As the concentration increased to 2 μM, the spontaneous transfer decreased by almost one-half. The concentration of donor vesicles and ROS membranes was the same as described in Fig. 1.
stopped flow experiments

Transfer of retinol between single unilamellar vesicles is rapid and independent of the concentration of donor and acceptor vesicles—In a typical experiment, the donor vesicles contained 0.1 mM PC, 5 mol % NBD-PE, and 3-5 mol % retinol, while acceptor vesicles contained 5-50 times the concentration of PC. There was a time-dependent increase in retinol fluorescence, indicating the movement of retinol from the donor (quenched) vesicles to the acceptor (unquenched) vesicles. At 22 °C, the first order rate constant for the kinetics of fluorescence increase was about 0.8 s⁻¹ in Tris/NaCl buffer. A trace of the fluorescence change reflecting this transfer process is shown in Fig. 6.

To determine whether this transfer was accomplished via the lipid phase or via the aqueous phase, we measured the transfer rates at different concentrations of donor and acceptor vesicles. If the transfer was accomplished via the lipid phase (during the collision of lipid vesicles), the transfer rate would increase as the concentration of donor or acceptor vesicles was increased. If transfer proceeded through the aqueous phase, the transfer rate would be independent of the concentrations of donor or acceptor vesicles. The results from this series of experiments are shown in Table I. Varying the concentration of acceptor vesicles 50-fold while the donor vesicles concentration was kept constant did not change the transfer rate. When the concentration of both donor and acceptor vesicles were varied 10-fold, the transfer rate remained constant. The presence of 2 μM IRBP as an additional acceptor for retinol did not change the transfer rate. It is clear from these results that the transfer rate is independent of the concentration of donor and acceptor vesicles. Therefore, we conclude that the spontaneous transfer of all-trans-retinol between SUV occurs via the aqueous phase.

Spontaneous transfer of retinol between single unilamellar vesicles is dependent on the physical state of donor vesicles and their environment—As shown in Fig. 7, the transfer rate whereas an identical concentration of IRBP reduced this transfer by only 5%. After the separation of ROS membranes, a portion of the supernatant was subjected to Sepharose CL-4B column. As in the previous experiment, two peaks were observed (Fig. 5). The first peak was liposomes, as judged by co-migration of ³H and ¹⁴C in a fixed ratio, and the second peak was BSA and its bound ligand, ³H-palmitic acid. In a similar experiment, IRBP bound much less palmitic acid than did BSA as evidenced by a much smaller second peak when compared to the BSA experiment (data not shown).
TABLE I

Effect of vesicle concentration on the transfer rate of retinol between SUV

Donor vesicles contained 5 mol % NBD-PE and 3 mol % all-trans-retinol. Acceptor was SUV alone or SUV plus 2 μM IRBP. The transfer was done at 22 °C in Tris/NaCl buffer. The experimental error was around 10%.

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<th>Donor vesicle concentration (mM)</th>
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<td>0.1</td>
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FIG. 7. Arrhenius plot of retinol transfer between single unilamellar vesicles in Tris/NaCl buffer. The activation energy (E_a), calculated from the Arrhenius equation (lnk = lnA - E_a/R; k, rate constant; R, gas constant; T, temperature (°K); A, constant), was 7.8 kcal/mol.

was temperature-dependent and was slower at lower temperatures. The activation energy of this transfer, calculated from the slope of the Arrhenius plot, was 7.8 kcal/mol. The transfer rate did not change as the concentration of retinol in the vesicles increased from 3 to 5 mol %. At 2 mol %, however, we did observe a slight decrease (about 20%) in transfer rate, although the signal-to-noise ratio decreased, and the variability between experiments increased at this low concentration of retinol. The rate of retinol transfer appeared to be an exponential function of ionic strength as higher concentrations of NaCl decreased the transfer rate significantly (Fig. 8). Addition of 40 mol % PE in the donor vesicles or lowering the buffer pH from 7.4 to 2.8 did not change the transfer rate.

Spontaneous Transfer of Retinol from IRBP to Single Unilamellar Vesicles Is Slower Than the Spontaneous Transfer between Single Unilamellar Vesicles—When retinol bound to IRBP moved to vesicles that contained NBD-PE, there was a time-dependent decrease in fluorescence. This transfer process had a half-time of approximately 6.3 s (Fig. 9), which was 8 times slower than the transfer between single unilamellar vesicles. Again, the transfer rate remained constant when acceptor vesicle concentration was varied 10-fold (data not shown).

To determine whether the fluorescence decrease we observed might be due to degradation of retinol, the following experiments were conducted. The absorbance of all-trans-retinol (at 326 nm) was monitored for 30 min when associated with liposomes, IRBP, BSA, or free in buffer. There was no change in absorbance over the 30-min period when retinol was associated with liposomes or IRBP. However, there was a 20% and 35% decrease in absorbance when retinol was associated with BSA or free in buffer, respectively (Fig. 10). Therefore, we conclude that the time-dependent decrease in fluorescence observed in the above experiments was due to...
retinol movement from IRBP to liposomes and not to the degradation of retinol.

**DISCUSSION**

The movement of retinoids between the photoreceptors and the retinal pigment epithelium in relationship to the light-dark cycle has been clearly established (24, 25), i.e. there is a net movement of retinol to the retinal pigment epithelium during light adaptation and a net movement of retinaldehyde to the ROS during dark adaptation. This movement takes place by way of the interphotoreceptor matrix, an extracellular aqueous compartment interposed between the photoreceptors and the retinal pigment epithelium. Since retinoids are high molecular weight glycoprotein in retinoid transport (1-4). Our studies clearly demonstrate that retinoids can rapidly move via the aqueous phase between membrane compartments. Thus, a transport protein is not required for retinoid movement across the interphotoreceptor matrix and the proposed role of IRBP in this process must be reconsidered.

Our equilibrium exchange experiments were initially designed to examine the kinetics of retinol transfer between liposomes and ROS and to determine whether IRBP could facilitate such transfer. The data from these experiments indicated that the spontaneous transfer of retinol occurred quickly, and that the rates were too fast to be determined with the methods employed. Neither was it possible to determine whether IRBP accelerated or retarded the transfer process, although IRBP did change the equilibrium distribution of retinol. In a series of similar transfer experiments using palmitic acid, similar observations were made, except that BSA changed the equilibrium substantially while IRBP did not.

In order to determine the mechanism and kinetics of retinol transfer, we used the method of fluorescence energy transfer and the stopped-flow device which can monitor the rapid, spontaneous transfer at millisecond intervals. We found that the transfer of retinol between single unilamellar vesicles occurred as a first order process with a half-time of 0.8 s, which was comparable to the exchange rate of medium chain fatty acids (8). The transfer rate was independent of the donor and acceptor concentration. A 50-fold variation in acceptor membrane concentration did not change the transfer rate. When the donor-to-acceptor ratio was kept constant, but their concentrations were varied 10-fold, the transfer rate remained unchanged. The transfer was also independent of the identity of the acceptor because addition of IRBP did not change the rate. These results satisfy the experimental criteria for a transfer mechanism occurring through the aqueous phase (8). Therefore, we conclude that the transfer of retinol between liposomes follows a mechanism similar to that of fatty acids and proceeds in two steps. First, retinol partitions between liposomes (or membranes) and the aqueous phase, followed by diffusion of the aqueous "soluble" retinol to the acceptor liposomes (or membranes).

Although we have not yet tested other retinoids, it is reasonable to expect that the transfer of retinaldehyde or retinoic acid follows a similar mechanism as we observed for retinol. Rando and Bangerter (15) examined the transfer of 11-cis-retinol and observed a rapid spontaneous transfer similar to that of all-trans-retinol. Pownall et al. (8) studied systemically the transfer of pyrene analogues of fatty acids, alcohols, and methyl esters, and concluded that their transfer followed a similar mechanism, but in different time frames depending on their chain length and polarity. Therefore, it is most likely that free retinaldehyde and retinoic acid also transfer via the aqueous phase but in a slightly different time frame because of their differences in polarity and charge. The transfer of retinyl esters (mainly retinyl palmitate and retinyl stearate in the retina) would be much slower because of their marked increased hydrophobicity.

Since our transfer data are derived from in vitro experiments, we can only speculate on their physiological significance. However, one can relate these data to the in vivo conditions by comparing the distance through which the exchange must take place. The average distance between lipid vesicles can be calculated as follows. Assuming that there are 2600 PC molecules in each vesicle (26), the number of vesicles will be about $2 \times 10^{16}$ per liter at a concentration of 0.1 mM PC. The average distance between each individual vesicle will be about 0.3 μm. The distance between the outer segment plasma membranes and the pigment epithelial apical microvilli that ensheath outer segments, as measured from published micrographs (27), is less than 0.1 μm. Therefore, the spacing between liposomes in our experiments is similar to the actual physical proximity of the plasma membranes of the cells bordering the interphotoreceptor matrix.

Another consideration is the possibility that the membrane compositions of either outer segments or pigment epithelium are so different that spontaneous transfer would not occur as in our experimental conditions. To test this, we have examined the spontaneous transfer of retinol from 100% PC liposomes versus 60% PC, 40% PE liposomes, and found no difference in the kinetics. Thus, while many other phospholipid combinations are possible in biological membranes, it is reasonable to propose that retinoid exchange between membranes with different lipid compositions can proceed through
the aqueous phase as occurred under our experimental conditions.

Our concept of spontaneous transfer of retinoids through the aqueous phase can be incorporated into the scheme of retinoid movement in the photoreceptor-pigment epithelium complex as follows. As a first step, retinoids reach equilibrium distribution in various retinal compartments by diffusion through the aqueous phase. Following the bleaching of rhodopsin in the light, all-trans-retinol concentration increases in the discs and plasma membranes of the outer segments and rapidly equilibrates between membranes and its binding proteins, namely, IRBP and CRBP. Therefore, a pool of retinol (either free retinol associated with membranes or that bound to CRBP) is readily available for esterification in the retinal pigment epithelium. In the dark, the 11-cis-retinaldehyde produced by retinal isomerase in the retinal pigment epithelium also rapidly partitions between membranes and its binding proteins, namely, IRBP and CRALBP. Therefore, a pool of 11-cis-retinaldehyde (either free retinaldehyde associated with ROS membranes or that bound to IRBP) will be readily available for rhodopsin regeneration. As a second step, retinoids are taken out of this equilibrium as they are sequestered in the pigment epithelium as retinyl esters, or as rhodopsin in the ROS. Through the equilibrium distribution and the sequestration processes, a net movement of retinol to the pigment epithelium occurs during light adaptation, and a net movement of retinaldehyde to the ROS occurs during dark adaptation.

The role of CRBP and CRALBP in retinoid transfer still must be defined. Our studies demonstrated that retinol transfers from IRBP to liposomes spontaneously with a half-time of 6.3 s. The spontaneous transfer of retinol from SRBP to liposomes has also been observed, although it is much slower than that from IRBP to liposomes (half-time about 30 min) (16). We speculate that the rate of such transfer is dependent on the relative affinities of these retinoid-binding proteins for their ligands. The kinetics of spontaneous transfer of ligands from CRBP or CRALBP, and the physiological role of these binding proteins are not known. It is possible that they may facilitate the intracellular processing of retinoids by regulating enzyme activity or targeting their ligands to specific membrane domains. Indeed it has been shown that apoCRBP facilitates the release of retinol from membrane-bound retinyl esters (28), and 11-cis-retinaldehyde bound to CRALBP and all-trans-retinol bound to CRBP may be the actual substrate for esterification in the pigment epithelium (29).

The observation that the endogenous isomer form of retinoids associated with IRBP varied in the light-dark cycle (i.e. IRBP isolated from light-adapted retinas carried more all-trans-retinol, while more 11-cis-retinaldehyde was associated with IRBP isolated from dark-adapted retinas) has often been cited as evidence of a role for this protein in retinoid transport (30, 31). These differences in endogenous ligands may merely reflect the change in equilibrium distribution of retinoids between IRBP, membranes, and other retinoid-binding proteins, which occurs during the light-dark cycle. In our experiments, we have demonstrated that IRBP changed the equilibrium distribution of retinol, and that the amount of retinol bound to IRBP was determined by the total amount of free retinol and the relative concentrations of IRBP and membranes. Therefore, the above observations of different ligands associated with IRBP in light versus dark can be explained as follows. In the light cycle, all-trans-retinol increases in the ROS and equilibrates between membranes, IRBP, and CRBP. Since CRBP has a much higher affinity for this ligand, it will always be saturated first, and then the amount of all-trans-retinol bound to IRBP will start to increase. Meanwhile, active regeneration of rhodopsin reduces the buffer of 11-cis-retinaldehyde bound to IRBP. In the dark cycle, IRBP readily releases its bound all-trans-retinol to the pigment epithelium and the amount of 11-cis-retinaldehyde bound to IRBP will increase after the saturation of CRALBP. The observed differences in endogenous ligands bound to IRBP therefore do not prove a transport role of IRBP in mediating retinoid movement, but actually reflect the different equilibrium distribution of retinoids in the light-dark cycle.

It is also clear from our studies that IRBP does not facilitate retinol transfer but actually retards the exchange of retinol between membranes. The amount of retinol transfer from liposomes to ROS was decreased by IRBP in equilibrium exchange experiments and the transfer of retinol from IRBP to single unilamellar vesicles is 8 times slower than the spontaneous transfer between single unilamellar vesicles. Based on these observations, the suggested role of IRBP as a retinoid-transport protein must be re-evaluated. A distinction must be made between a protein that facilitates the transport of a molecule across a membrane or aqueous compartment, and a protein that binds a ligand but does not have an active role in its transport. In this context, IRBP can only be regarded as a protein which binds retinoids, but does not facilitate the transport of retinoids across the interphotoreceptor matrix.

IRBP, however, may serve other important functions in the interphotoreceptor matrix. We have demonstrated that IRBP effectively protects retinol from degradation, similar to what has been documented for SRBP (32). In this regard, IRBP can serve as a reservoir for retinoids in the interphotoreceptor matrix. Another possible function of IRBP would be to protect membranes from damage caused by rapid accumulation of retinoids following a strong bleach. Since retinoids, as well as free fatty acids, are notorious for their membranolytic effects (33), a binding protein like IRBP may be necessary to protect membranes from such damage by allowing the gradual exchange of retinoids. The finding that 1 molecule of IRBP also binds 4 molecules of fatty acids (34) lends further support to this putative role. In this role, IRBP may be considered as an important buffer for retinoids such as of extensive bleaching.

In summary, we have provided evidence that the transfer of retinol between membranes proceeds through the aqueous phase and does not require a transport protein. Based on our observations, we conclude that IRBP does not facilitate the transport of retinoids, but acts passively by allowing the gradual exchange of retinoids. However, it is also likely that other important functions of IRBP may be uncovered in the future if we are no longer conditioned by the concept of its role in retinoid transport.

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REFERENCES
Movement of Vitamin A