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MINI-REVIEW

Membrane Phospholipids and the Dark Side of Vision

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Abstract

The key step in the visual pigment regeneration process is an enzyme-catalyzed *trans* to *cis* retinoid isomerization reaction. This reaction is of substantial general interest, because it requires the input of metabolic energy. The energy is needed because the 11-*cis*-retinoid reaction products are approximately 4 kcal/mol higher in energy than their all-*trans* congeners. In the retinal pigment epithelium a novel enzymatic system has been discovered which is capable of converting all-*trans*-retinol into all-*trans* retinyl esters, by means of a lecithin retinol acyl transferase (LRAT), followed by the direct processing of the ester into 11-*cis*-retinol. In this process the free energy of hydrolysis of a retinyl ester, estimated to be approximately -5 kcal/mol, is coupled to the endothermic ($+4$ kcal/mol) isomerization reaction, resulting in an overall exothermic process. The overall process is analogous to ATP-dependent group transfer reactions, but here the energy is provided by the membrane phospholipids. This process illustrates a new role for membranes: they can serve as an energy source.

Key Words: Visual cycle; membrane; phospholipids; isomerohydrolase; isomerase; lecithin retinol acyl transferase; vitamin A.

Introduction

Phospholipid-based membranes have two well-known functions. In their classical bilayer form, their primary function is to demark the inside from the outside of a cell. The hydrophobicity of the fatty-acyl chains of the phospholipids prevents the random translocation of hydrophilic materials into

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and out of the cell. A second major function of membranes, discovered more recently, is to serve as a source of second messenger molecules. For example, the phospholipase A_2-mediated hydrolysis of phospholipids at the *sn*-2 position produces unsaturated fatty acids, which can be oxidatively processed to form the various prostaglandins, prostacyclins, thromboxanes, and leukotrienes which are mediators of diverse biological phenomena (Samuelsson, 1980). Our understanding of this second function has been advanced by the discovery that trace membrane lipids, such as the polyphosphoinositides, are also processed by specific phospholipases to afford polyphosphoinositides (Berridge, 1981) and diacylglycerides (Nishizuka, 1984), both of which also have important second messenger functions. These two well-known views of membrane function serve as a backdrop for a third function of membranes: they may also serve as an energy source (Deigner *et al.*, 1989). This new perspective on membrane function is the subject of this review.

It is generally understood that phosphate esters are the energy currency of cells. The salient issue here is the large negative free energies of hydrolysis of these compounds. For example, the free-energy change for the ATP + $H_2O \rightarrow$ ADP + P_i + H^+ transformation is -7.3 kcal/mol (Jencks, 1970). The imprecise term "high-energy bond" is often used to describe this free-energy change. Energy transduction mechanisms in cells generally involve the use of mechanisms that couple hydrolytic free-energy changes of phosphate esters to an otherwise endothermic process. This serves as the basis of group-transfer processes in biochemistry. For example, the endothermic biosynthesis of sucrose from glucose and fructose, shown in Fig. 1, involves the intermediate formation of phosphorylated glucose, which is then condensed with fructose to form the product sucrose. The overall free-energy change is the sum of the two independent reactions. Thus, endothermic disaccharide formation is made possible by the negative free energy of hydrolysis of ATP via the group transfer process.

In principle, a similar situation is possible using acyl esters as an energy source. This is because esters, such as ethyl acetate, are hydrolyzed with ΔG^0s in the -5 kcal/mol range (Jencks, 1970). Thus, if acyl ester moieties could be transferred from donor to acceptor molecules, they could be used in ways similar to the phosphate group. In this article a process will be described in which an acyl group is transferred from the *sn*-1 position of phosphatidylcholine (lecithin) to all-*trans*-retinol (vitamin A) to form retinyl esters. This reaction is catalyzed by lecithin retinol acyl transferase (LRAT). In the second part of the process, the all-*trans*-retinyl ester is directly isomerized into 11-*cis*-retinol with the concomitant hydrolysis of the acyl ester. This hydrolysis provides the necessary energy to drive the otherwise endothermic *trans* to *cis* isomerization reaction. Lecithin serves as an ATP surrogate in this overall process, since the acyl group transfer from lecithin to all-*trans*-retinol

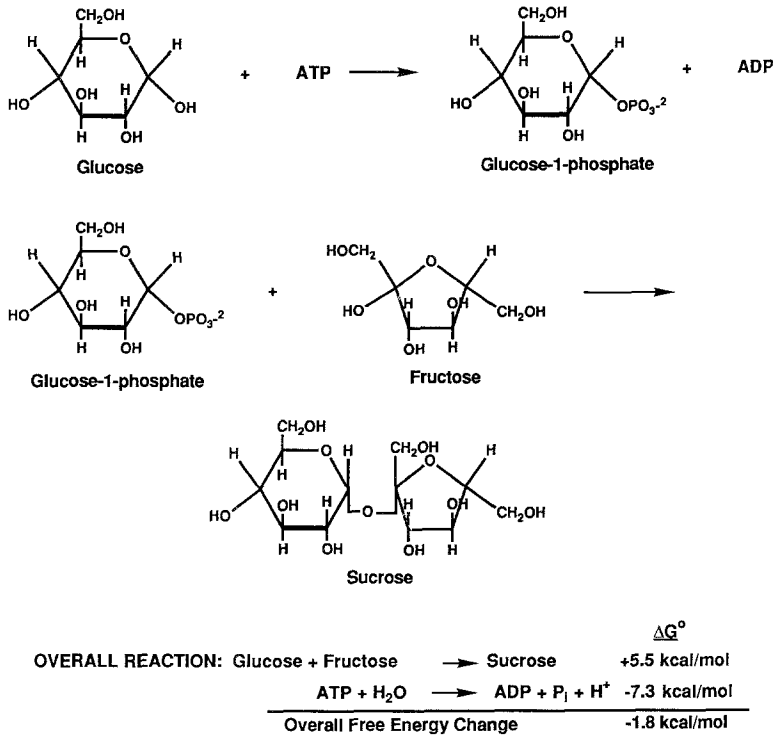


Fig. 1. Biosynthesis of sucrose using ATP.

activates the latter and provides the energy required to allow for the formation of 11-*cis*-retinol. The enzyme-catalyzed oxidation of 11-*cis*-retinol to 11-*cis*-retinal in the eye provides the chromophore required for the formation of the visual pigment rhodopsin. In the next section, a brief description of rhodopsin and the visual cycle will be presented, in order to provide a context for subsequent discussion of the energy-requiring biosynthesis of 11-*cis*-retinol.

A Photochemical *cis* to *trans* Isomerization Initiates Vision

Rhodopsin is an integral membrane-bound protein containing 11-*cis*-retinal covalently linked to its active-site lysine by means of a protonated Schiff base (Bownds, 1967). The absorption of a photon of light by vertebrate rhodopsin leads to the *cis* to *trans* isomerization of the chromophore, and the eventual hydrolysis of the all-*trans*-retinal Schiff base, in a process called bleaching (Hubbard and Wald, 1952). A conformational intermediate(s)

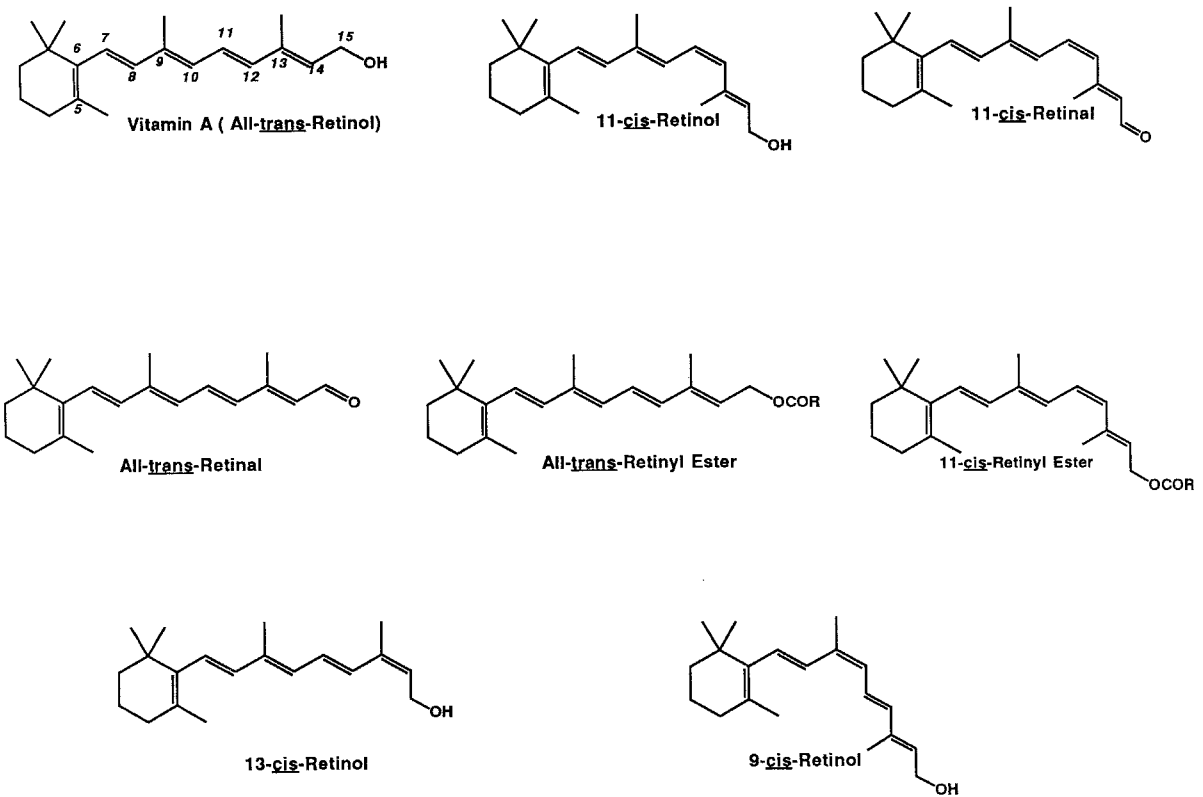


Fig. 2. Structures of vitamin A and other retinoids.

of photolyzed rhodopsin, which occurs prior to bleaching and which is spectroscopically defined as metarhodopsin II (Longstaff *et al.*, 1986), catalyzes the exchange of GTP for GDP in the retinal G-protein (Stryer, 1986). This initiates the visual transduction cascade in the vertebrate retina, which ultimately results in the closing of the rod outer segment plasma membrane sodium channels (Stryer, 1986; Wheeler and Bitensky, 1977). The closing of these *c*GMP-regulated channels causes the hyperpolarization of the rods and the initiation of the neural response of vision (Fesenko *et al.*, 1985). In order for vision to proceed, the visually active chromophore 11-*cis*-retinal (Fig. 2) must be resynthesized in the eye, either directly or indirectly, from an all-*trans*-retinoid precursor in the dark (Hubbard and Wald, 1952). The bleaching of rhodopsin and the subsequent reprocessing of all-*trans*-retinoids to the 11-*cis*-retinal used for recombination with opsin are referred to as the vertebrate *Visual cycle*. The biosynthesis of 11-*cis*-retinal is of critical importance if vertebrate vision is not to have the character of a once-in-a-lifetime event.

A particularly vexing problem in visual science has been the thermal mechanism by which the 11-*cis*-retinal is reformed in the vertebrate eye. The eye is the only organ in the body in which this isomeric form of retinoid is found to a significant extent. As of 1986, no system able to generate 11-*cis*-retinoids *in vitro* in darkness had yet been confirmed.

How then does the biosynthesis of 11-*cis*-retinal occur in the eye? The next section will elucidate the nature of this process as revealed by recent evidence.

The Visual Cycle

As stated above, all-*trans*-retinal is released from rhodopsin in the retina after bleaching. It is then rapidly reduced to form all-*trans*-retinol (vitamin A) (Fig. 2) in the rod outer segment proper by one of the retinol dehydrogenases (Wald and Hubbard, 1949). Under bright light conditions, the resulting vitamin A is then bound to a binding protein(s) (Pfeffer *et al.*, 1983; Saari *et al.*, 1984; Bok, 1985; Adler and Evans, 1985) and transported to the pigment epithelium, where it is esterified to long-chain fatty acids and stored (Hubbard and Dowling, 1962; Knowles and Dartnall, 1977). (The pigment epithelium is the organ that sits behind the retina in the eye.) For each all-*trans*-retinoid, there is a corresponding 11-*cis*-retinoid, and the totality of biochemical reactions required to interconvert these molecules comprises the classical and oversimplified visual cycle (Fig. 3). Thus, the double-bond isomerization reaction could potentially occur with any of three substrates and produce any of the three products, and it could occur either in the pigment epithelium or in the retina. After isomerization has occurred, the

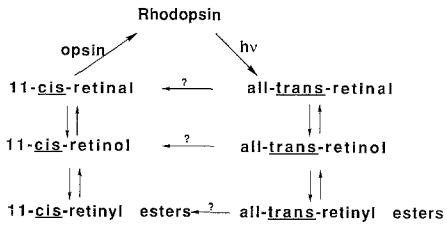


Fig. 3. The classical visual cycle.

11-*cis*-retinoid, either in the alcohol or aldehyde form, must be transported back to the rod outer segments by means of specific binding proteins (Saari *et al.*, 1984; Bok, 1985). There it can be used for rhodopsin regeneration (after oxidation, if 11-*cis*-retinol is transported back).

The dynamic nature of the visual cycle can be understood from experiments designed to measure the various retinoids during dark and light adaptation in living animals (Hubbard and Dowling, 1962; Knowles and Dartnall, 1977). In the dark, the retinoid pool in an animal such as the frog can consist of up to 75% 11-*cis*-retinoids, of which 11-*cis*-retinal bound to opsin in the retina and 11-*cis*-retinyl esters in the pigment epithelium are the major components. In the light, there is a major shift to the all-*trans*-retinoid congeners, with the esters being the predominant form, while dark adaptation again reverses this process (Hubbard and Dowling, 1962). Since none of the enzymes of the visual cycle has as yet been completely purified or characterized, possible elements of control remain unidentified.

Before proceeding further it is important to note that at chemical equilibrium 11-*cis*-retinoids comprise approximately 0.1% of the equilibrium mixture, and hence there is a more than 4 kcal/mol difference between a given 11-*cis* isomer and its all-*trans* counterpart (Rando and Chang, 1983). Therefore, in addition to identifying the components involved in the isomerization process, a central question that needs to be addressed is where the energy comes from to drive the *trans* to *cis* isomerization process. Part of this energy source could be derived from the binding interactions of retinoids with proteins specific for the 11-*cis* configuration of retinoids, but as noted above, up to 75% of the retinoids in a dark-adapted animal are in the 11-*cis* form, far exceeding the amount of 11-*cis*-retinal bound to rhodopsin or 11-*cis*-retinoid binding proteins (Saari and Bredberg, 1987). The bulk of the remaining 11-*cis*-retinoids is present as 11-*cis*-retinyl palmitate in an unbound form in the pigment epithelium. Moreover, the addition *in vivo* of aromatic amines, which catalyze the thermal isomerization of 11-*cis*-retinal back to all-*trans*-retinal, significantly depletes stores of all 11-*cis*-retinoids in the

dark-adapted eye (Bernstein *et al.*, 1986). This result is not compatible with the presence of large enough quantities of specific retinoid binding proteins to strongly shift the equilibrium toward the 11-*cis*-retinoids. It is clear that an additional energy input beyond specific binding interactions is required in order to account for the nonequilibrium nature of the isomeric retinoid pools in the dark-adapted eye. This issue of where the energy comes from that provides the driving force for the *trans* to *cis* retinoid isomerization is the central topic of this review.

11-*cis*-Retinoid Regeneration *in vitro*

In 1986 we were able to demonstrate the first *in vitro* system able to transform an all-*trans*-retinoid into an 11-*cis*-retinoid (Bernstein *et al.*, 1987a). A 600-g supernatant membrane preparation from amphibian retina/pigment epithelium proved capable of converting added all-*trans*-retinol to a mixture of 11-*cis*-retinol, 11-*cis*-retinal, and 11-*cis*-retinyl palmitate (Bernstein *et al.*, 1987a). All-*trans*-retinyl palmitate and all-*trans*-retinal were also generated by these membranes. A time-dependent, approximately linear increase in the absolute amount of 11-*cis*-retinol formed was found, while in the absence of eye tissue none was produced (Bernstein *et al.*, 1987a). As would be expected of a biologically significant process, the membrane-bound 11-*cis*-retinoid biosynthetic activity proved to be heat sensitive (Bernstein *et al.*, 1987a). Intact protein and membrane are required, because activity was abolished by treatment with proteinase K or phospholipase C (Bernstein *et al.*, 1987b).

The results described above unequivocally demonstrate the presence of an 11-*cis*-retinoid biosynthetic activity which was able to produce substantial quantities of 11-*cis*-retinoids. For example, 11-*cis*-retinol could be up to 40–50% of the isolated retinol pool. This figure is far removed from the equilibrium value. It was of interest to determine whether the isomerase activity is located in the retina or in the pigment epithelium, and to determine the precise nature of the substrate for isomerization. Studies on the amphibian showed that the isomerase activity was almost entirely located in the membranes of the pigment epithelium (Bernstein *et al.*, 1987a). The trivial amount of activity found in the retinal fraction was almost certainly due to contamination by a small amount of adherent pigment epithelium. The isomerization activity has also been observed in the bovine system, where a more complete separation of the retina from the pigment epithelium is possible, and here the activity was found only in the pigment epithelium membranes (Fulton and Rando, 1987).

Substrate Specificity of the Isomerase

Since labeled all-*trans*-retinol can be converted into all-*trans*-retinal, all-*trans*-retinyl palmitate, and all three of their 11-*cis* congeners by the pigment epithelium membrane preparation described, it cannot be assumed that all-*trans*-retinol is the substrate for the isomerase or that 11-*cis*-retinol is the direct product of the isomerase's action. However, the proposition that free all-*trans*-retinal cannot be the isomerase substrate was directly demonstrated by the finding that [15-^{3}H-15-^{14}C]-all-*trans*-retinol is processed to 11-*cis*-retinol by the native frog and bovine pigment epithelium membranes with complete retention of the tritium label (Bernstein *et al.*, 1987b). Had obligate oxidation to the retinal occurred, loss of one-half of the ^{3}H in all of the synthesized 11-*cis*-retinoids would have resulted. This result is completely consistent with an *in vivo* variant of this double-label experiment performed earlier (Bernstein and Rando, 1986), and rules out the possibility that all-*trans*-retinal is the isomerase substrate.

The experiments described above show that retinal cannot be the substrate, but they do not distinguish between retinol and retinyl palmitate as possible substrates. When all-*trans*-retinol is processed by bovine pigment epithelium membranes, virtually all of the retinol is rapidly esterified to form all-*trans*-retinyl esters (Deigner *et al.*, 1989). Moreover, 11-*cis*-retinol is produced concomitant with the decrease in the levels of the all-*trans*-retinyl esters. Finally, when all-*trans*-retinyl palmitate is processed by these membranes, 11-*cis*-retinol is produced (Deigner *et al.*, 1989). Although these experiments taken together do not unequivocally prove the point, they strongly suggest that retinyl esters may be the actual isomerase substrate and that the esters are directly converted into 11-*cis*-retinol. The ramifications of this kind of process will be discussed later, when energetic questions are addressed. The nature of the substrate for the isomerase turns out to be inextricably linked to the nature of the energy source.

The specificity of the isomerase system toward retinol isomers is considerable. In washed pigment epithelium membranes, added 9-*cis*- and 13-*cis*-retinol are efficiently esterified; however, neither is isomerized to a significant extent (Bernstein *et al.*, 1987b). Methylated analogs of all-*trans*-retinol, such as the *O*-methyl and 15-methyl derivatives, have been studied. The *O*-methyl derivative is completely inactive, whereas the 15-methyl derivative is an exceedingly weak substrate for the isomerase (Law and Rando, unpublished data). Thus, the isomerase system appears to be highly specific for all-*trans*-retinoids.

Structure–activity studies were also performed on a variety of dihydroretinols (Law *et al.*, 1988b). None of the dihydro isomers was substantially processed into its 11-*cis* counterpart, although vitamin A_2 was. Vitamin A_2

contains a second double bond in the cyclohexyl moiety (Law *et al.*, 1988b). Thus, an intact polyene chain is essential if a molecule is to be an isomerase substrate (Law *et al.*, 1988b). The mechanistic ramifications of this will be discussed later. It is interesting to note that vitamin A_2, the visual chromophore in early amphibian development, was processed by the isomerase system (Law *et al.*, 1988b).

Biochemical Characterization of the Isomerase System

As already mentioned, the isomerase system is membrane bound. The isomerase activity can be removed from the supernatant by centrifugation, with the concomitant appearance of the activity in the pellet (Bernstein *et al.*, 1987a). Phospholipase C treatment irreversibly abolishes the activity of the enzyme(s), as does treatment with several detergents (Bernstein *et al.*, 1987b). In addition, high concentrations of salt (KCl) or a chelating agent (EDTA) do not render the enzyme(s) soluble, suggesting that the isomerase is an integral membrane component, rather than being membrane associated (Bernstein *et al.*, 1987b). The isomerase activity also appears to distribute, without great preference, throughout the various membrane fractions, as determined by centrifugation studies (Fulton and Rando, 1987). The isomerases from both bovine and amphibian pigment epithelial membranes have K_M's in the 0.5–1 μM range. In the case of bovine tissue, V_{max} = 0.5 nmol/h/mg protein. Rates of this magnitude can account for the endogenous rates of rhodopsin regeneration *in vivo*.

Detergent Solubilization and Partial Purification of the Isomerase and Ester Synthetase

As mentioned above, the isomerase and ester synthetase enzymes are quite sensitive to a wide variety of detergents (Bernstein *et al.*, 1987b). However, we have recently been able to solubilize both activities successfully in the zwitterionic detergent Zwittergent-3,14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) (Barry *et al.*, 1989). Three initial criteria for solubilization were used. First, high-speed centrifugation ($> 150{,}000\,g$) left the activities in the supernatant. Second, the solubilized activities were partitioned between the void volume and the included volume upon gel filtration. Finally, the enzymatic activities were quantitatively passed through a 0.22-μm filter. Using anion exchange and gel filtration chromatography, the ester synthetase was purified approximately 200-fold (Barry *et al.*, 1989). The

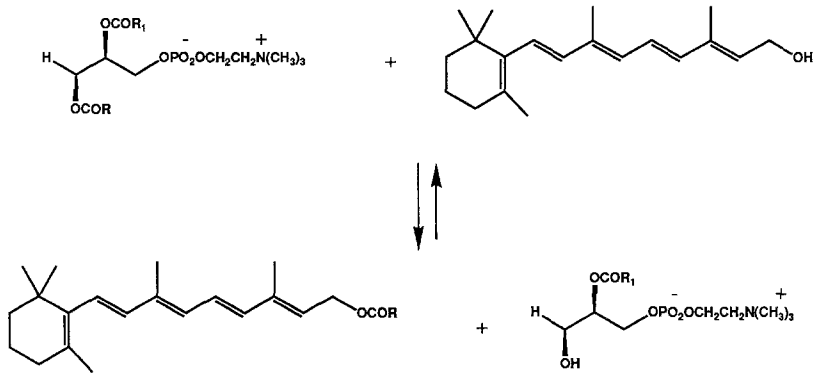


Fig. 4. Transesterification of retinol with phosphatidylcholine (lecithin) catalyzed by the enzyme LRAT.

isomerase was purified approximately 10–14-fold after anion-exchange chromatography (Barry *et al.*, 1989).

Several interesting observations were made on the enzymes as a consequence of this work. It was discovered that the acyl donor for retinol ester synthesis is lecithin, and that the transfer occurs from the 1-position of the phospholipid (Fig. 4) (Barry *et al.*, 1989). The enzyme in question is a lecithin retinol acyl transferase (LRAT). Similar observations were made by Saari and Bredberg (1989). Thus, the retinyl ester synthetase operates by a transesterification route similar to that found in the liver (MacDonald and Ong, 1988).

Membranes as the Energy Source for Isomerization

An intriguing relationship exists between endogenous LRAT and isomerization which provides the key to the nature of the energy source (Fulton and Rando, 1987). Thus far, studies on the membrane localization of the isomerase and LRAT activities have shown that the two activities cannot be completely dissociated from one another either by differential or density gradient centrifugation studies (Fulton and Rando, 1987; Bernstein *et al.*, 1987b). As mentioned above, the solubilized enzymatic activities also appear to copurify (Barry *et al.*, 1989). Chemically diverse reagents, such as ethanol, hydroxylamine, and *p*-hydroxymercuribenzoate, all appear to inhibit both activities in a roughly parallel manner (Fulton and Rando, 1987). However, to directly and unequivocally determine whether or not retinyl esters are the isomerase substrates, a specific inhibitor of LRAT was required.

All-*trans*-retinyl-α-bromoacetate is a potent and specific competitive inhibitor of LRAT (Trehan *et al.*, 1990). In the micromolar range this

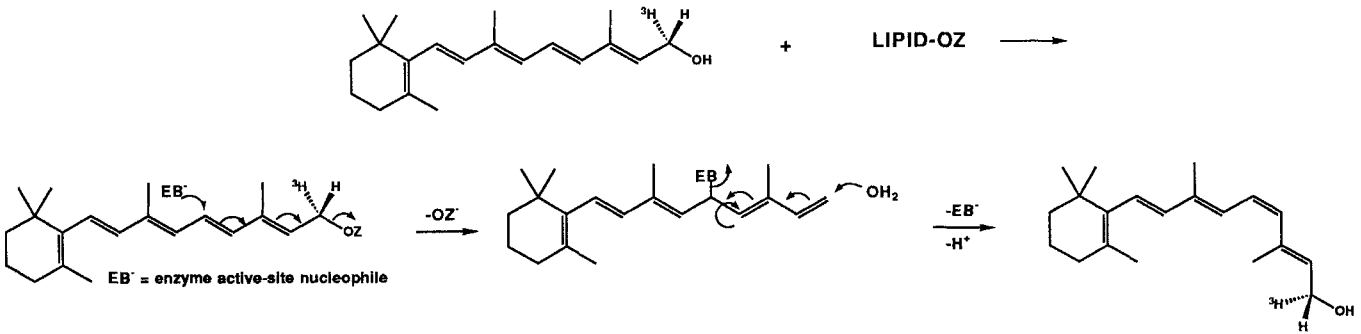
inhibitor abolishes LRAT activity and also prevents 11-*cis*-retinol formation from all-*trans*-retinol (Trehan *et al.*, 1990). Most significant, however, is the finding that all-*trans*-retinyl esters are readily processed directly into 11-*cis*-retinol in the presence of the inhibitor (Trehan *et al.*, 1990). These experiments unambiguously demonstrate that all-*trans*-retinyl esters are obligate in the biosynthesis of 11-*cis*-retinol.

Why might the linkage of ester synthesis to isomerization be of interest? Esters are "high-energy" compounds and are hydrolyzed with free energies of hydrolysis in the -5 kcal/mol range (Jencks, 1970). If the free energy of hydrolysis of an ester could be coupled to the isomerization process, more than enough energy would be provided to drive the latter process. A possible mechanism by which ester hydrolysis could be linked to isomerization is shown below (Fig. 5) (Deigner *et al.*, 1989; Barry *et al.*, 1989). Here, the substrate would be an all-*trans*-retinyl ester, and the product, 11-*cis*-retinol. The enzyme that carries out this transformation is an isomerohydrolase rather than a simple isomerase. This isomerization reaction can be broken down into two independent reactions as shown in this scheme. The overall free-energy change is the sum of the two component reactions, resulting in an exothermic isomerization (Fulton and Rando, 1987; Deigner *et al.*, 1989; Barry *et al.*, 1989). Most interestingly, the energy to drive the reaction would originate in the membrane phospholipids, since retinyl esters arise by a transesterification reaction with lecithin (Barry *et al.*, 1989). Thus, in this case the phospholipids would act as an ATP surrogate.

The mechanism shown in Fig. 5 makes testable predictions. One prediction is that C–O bond cleavage accompanies isomerization. Furthermore, this cleavage could have an effect on the stereochemistry at C-15, since the prochiral center becomes symmetrical at the intermediate state. The prediction of C–O bond cleavage was readily testable by studying the fate of the ^{18}O of [15-^{18}O]-all-*trans*-retinol after isomerization in [^{16}O]-H_2O. This experiment demonstrated unequivocally that the 11-*cis*-retinol product formed contained only ^{16}O (Deigner *et al.*, 1989). Hence, complete cleavage and replacement of the C–O bond accompanies isomerization.

Stereochemical experiments were also performed on chirally labeled R and S [15-^{3}H]-all-*trans*-retinol (Law *et al.*, 1988a). It was found that complete inversion of configuration occurred during isomerization, a result only understandable if C–O bond cleavage had occurred (Deigner *et al.*, 1989).

The stereochemical and isotopic release studies described above entirely support the mechanism shown in Fig. 5. Most importantly, this mechanism provides a solution to the issue concerning the nature of the energy source used to drive the isomerization reaction and could be of general interest as a new mechanism of energy transduction in membranes (Fig. 6). In this mechanism, phospholipids function similarly to the way ATP does in the



			ΔG^0
Overall Reaction:	trans-Retinyl Ester + H_2O	trans-Retinol	-5 kcal/mol (approx.)
	trans-Retinol	11-cis-Retinol	+4 kcal/mol
	trans-Retinyl Ester + H_2O	11-cis-Retinol	-1 kcal/mol (net)

Fig. 5. A coupled reaction can drive the thermodynamically unfavorable isomerization reaction.

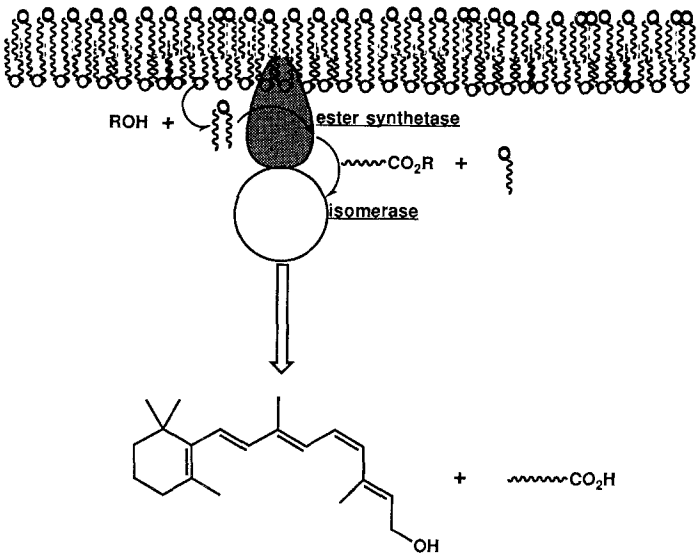


Fig. 6. The biosynthesis of 11-*cis*-retinol showing the involvement of membrane phospholipids.

formation of sucrose from glucose and fructose (Fig. 1). That is, the group transfer of an acyl group from lecithin to retinol bears similarity to the transfer of a phosphate group from ATP to glucose, in that in both cases this transfer produces an activated species with sufficient energy to produce the desired product. Also in both cases, the salient energy-producing reactions are hydrolysis reactions.

Returning to the nature of the visual cycle, our updated understanding of the cycle is shown in Fig. 7. This figure shows the direct isomerization of an all-*trans*-retinyl ester into 11-*cis*-retinol via an isomerohydrolase enzyme. Previously, retinyl esters were considered to play a passive role in the visual cycle in that they were thought to serve only as chemically inert storage forms of vitamin A. In our current formulation, retinyl esters play a critical and dynamical role in the biosynthesis of 11-*cis*-retinol.

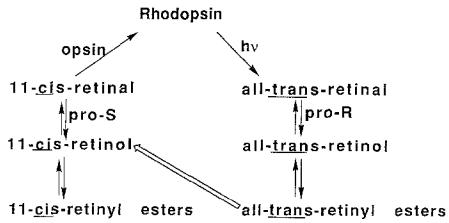


Fig. 7. Current model of the visual cycle.

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