Biosynthesis of 11-*cis*-Retinoids and Retinyl Esters by Bovine Pigment Epithelium Membranes†

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ABSTRACT: Previously, we have shown that retina/pigment epithelium membranes from the amphibian can synthesize 11-*cis*-retinoids from added *all-trans*-retinol [Bernstein, P. S., Law, W. C., & Rando, R. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1849-1853]. The activity was largely localized to the pigment epithelium. Here it is shown that, in the bovine system, the activity resides *exclusively* in the membranes of the pigment epithelium. Subcellular fractionation does not reveal a particular organelle where the activity resides. Washed bovine pigment epithelium membranes, which are devoid of retinoid redox activity, convert added *all-trans*-retinol to a mixture of 11-*cis*-retinol and its palmitate ester. *all-trans*-Retinal and *all-trans*-retinyl palmitate are not converted into 11-*cis*-retinoids by the membranes. The membranes show substantial ester synthetase activity, producing large amounts of *all-trans*-retinyl palmitate. Diverse chemical reagents, such as ethanol, hydroxylamine, and *p*-(hydroxymercuri)benzoate, inhibit both ester synthetase and isomerase activities in a roughly parallel fashion, suggesting a possible functional linkage between the two activities.

The absorption of light by rhodopsin results in the cis to trans isomerization of rhodopsin's 11-*cis*-retinal Schiff base chromophore (Hubbard & Wald, 1952). The *all-trans*-retinal Schiff base is hydrolyzed, producing *all-trans*-retinal and opsin, in a process called bleaching (Bownds, 1967). In order for vision to proceed, 11-*cis*-retinal must be biosynthesized in the eye by a purely thermal pathway. The biochemical reactions that make up this pathway are said to comprise the "visual cycle", and the functioning of this cycle is critical to the process of dark adaptation whenever substantial bleaching has occurred (Knowles & Dartnall, 1977a).

The last several years have seen much progress made toward a molecular understanding of visual transduction—a process which is comprised of those biochemical events connecting the absorption of a quantum of light by rhodopsin and the closing of the rod outer segment plasma membrane sodium channels (Fung & Stryer, 1980; Wheeler & Bitensky, 1977; Fesenko et al., 1985). By contrast, very little progress has been made toward either a molecular or a physiologic understanding of the visual cycle. None of the enzymes has been purified nor have any elements of control been identified. Central to an understanding of this cycle is the unravelling of the trans to cis isomerization process. More than a simple isomerization of *all-trans*-retinal to its 11-cis congener is required here, because of the endergonic nature of the process (Rando & Chang, 1983). In a dark-adapted eye, greater than 75% of the retinoids are 11-cis, whereas at chemical equilibrium only 0.1% of them are. In addition, other retinoids are part of the cycle, so that the substrate for the isomerization process need not be free *all-trans*-retinal and, in fact, is not (Bernstein & Rando, 1986). The *all-trans*-retinal produced from the bleaching process is rapidly reduced in the retina to *all-trans*-retinol, which is then transported to the adjoining pigment epithelium, where it is esterified and stored as long-chain fatty acid esters (Knowles & Dartnall, 1977a).

The isomerization process which closes the cycle has eluded even in vitro detection until very recently, when it was shown that frog pigment epithelium membranes can process added *all-trans*-retinol to produce a mixture of 11-*cis*-retinoids (Bernstein et al., 1987a). It is of great importance to an understanding of vision to characterize this isomerization process on a molecular level. The process also promises to be quite unique on a chemical level, because, as mentioned above, the isomerization process is endergonic in nature and hence may involve coupled reactions.

Before a molecular understanding of this isomerization process can be reached, the components need to be purified and characterized. To these ends, it is reported here that the isomerase is also found in the bovine eye, demonstrating the existence of the isomerizing system in the mammalian eye as well as in the amphibian eye, and making way for the eventual purification of the system because of the ready access to large amounts of the bovine material. In addition, the isomerase is partially characterized and is found to be located exclusively in pigment epithelium membranes. Only *all-trans*-retinol will serve as substrate for the putative enzyme(s). However, the isomerase, or 11-*cis*-retinoid synthetase, also appears to be linked in an as yet undetermined manner to ester synthetase activity. Both activities are broadly distributed in the membranes and show parallel sensitivities to a variety of chemical reagents. Possible reasons for this are discussed.

Materials and Methods

Materials

Frozen eye cups devoid of retinas were obtained from J. A. and W. L. Lawson Co., Lincoln, NE. Fresh calf eyes were obtained from a local slaughterhouse. [11,12-^{3}H]-*all-trans*-Retinol (53 Ci/mmol, >95% pure) was obtained from Amersham Inc. [11,12-^{3}H]-*all-trans*-Retinal and [11,12-^{3}H]-*all-trans*-retinyl palmitate were prepared by standard methods from [11,12-^{3}H]-*all-trans*-retinol (Bridges & Alvarez, 1982). Standard mixtures of the isomeric retinols, retinals, and retinyl palmitates were prepared by routine methods as previously described (Bernstein et al., 1986). Hydroxylamine, sodium

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iodoacetate, fatty acid free bovine serum albumin (BSA), and *p*-(hydroxymercuri)benzoate were products of Sigma Chemical Co. ScintiLene was a product of Fisher Chemical Co. 5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from Fluka Chemical Co.

Methods

Preparation of the Isomerizing System from Bovine Pigment Epithelium. All preparations were conducted in the cold room under normal light unless otherwise noted. All experiments performed with added retinoids were done in the cold room or on ice, in both cases under a dim red light; 25 frozen bovine eye cups obtained from J. A. Lawson Co. (Lincoln, NE) were thawed in a container immersed in warm water over a period of 2–3 h. The eye cups were placed on a bed of ice, and 2 mL of 0.32 M sucrose/0.1 M (sodium/potassium) phosphate buffer at pH 7.4 was added. The retinal pigment epithelium cells were brushed into the buffer with a no. 2 camel hair brush. The resultant ice-cold cell suspension (40–50 mL) was vigorously disrupted with a Branson sonifier cell disruptor (Model W185) by three 30-s bursts with a 30-s cooling period between bursts. The membrane components were obtained by centrifuging the burst cells in a swinging-bucket rotor at 1500*g* for 15 min at 5 °C. The red supernatant was removed, and the black pellet was resuspended in 5 mL of the buffered sucrose and recentrifuged at 1500*g*. The pellet was discarded, and the supernatants were combined and centrifuged at 150000*g* in a Beckman 50.2 Ti rotor for 75 min at 5 °C. The cytosol was discarded, and the pellet was washed with 0.1 M phosphate buffer at pH 7.4. The wash was discarded, and the pellet was suspended in 5.0 mL of the phosphate buffer and dispersed with an ice-cold Teflon/glass homogenizer. The protein concentrations were measured by using the Peterson modification of the Lowry method (Peterson, 1977). The membrane preparation was then divided into 0.5-mL aliquots, frozen in liquid nitrogen, and stored at −80 °C. The inclusion of a variety of protease inhibitors such as phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, and aprotinin in the buffers did not appear to enhance the stability of the isomerizing system substantially. Brief heating at 100 °C prevented isomerization. Treatment of the isomerase-containing membranes with proteinase K also inhibited isomerization. It should also be noted that the washing procedures used above deplete the membranes of redox activity, because the nicotinamide adenine dinucleotide cofactors are removed. This simplifies product analysis because it prevents the retinol to retinal interconversion.

In a typical preparation, the 150000*g* pellet contained 3.14 mg of protein/mL in a total of 5 mL, affording 15.7 mg of protein. This was enough material for approximately 150 assays. The discarded supernatant fraction contained 156.0 mg of protein in a total volume of 48 mL.

Assay of Isomerizing Activity. Unless otherwise noted, all assays were performed in the absence of light using the washed membrane preparation described above. Assays were conducted in a manner virtually identical with that already published (Bernstein et al., 1986). [11,12-^{3}H]-*all-trans*-Retinol was made up in a stock solution at 10 μM with a specific activity of 1 mCi/μmol. Under dim red light, 20 μL of the retinol solution was placed in a 1.5-mL plastic disposable centrifuge tube. The ethanol was evaporated with a stream of nitrogen, and 10 μL of freshly prepared 0.1 mg/μL defatted bovine serum albumin dissolved in 0.1 M phosphate buffer was added. Phosphate buffer and 0.1 mg of the membrane preparation containing the isomerizing activity were added to produce a final volume of 0.2 mL. The final solution was 1.0 μM in retinol (0.2 μCi) and 75 μM in BSA. After incubation at 38 °C for 30 min, 0.5 mL of methanol was added to stop the reaction. The mixture was vortexed, 0.4 mL of hexane containing 1 mg/mL butylated hydroxytoluene (BHT) was added, and the mixture was vortexed again for 1 min before centrifuging in a Beckman microfuge at 12000 rpm at 5 °C for 5 min. A control tube containing no eye tissue, but otherwise identical, was prepared for each assay. All assays were generally performed in duplicate.

Table I: Chemical Esterification of HPLC-Purified Retinols[a]

retinol isomer	isomeric composition of retinyl palmitates			
	% 11-cis	% 13-cis	% 9-cis	% all-trans
11-*cis*-retinol	74.9	12.2	2.5	10.4
13-*cis*-retinol	4.4	88.6	1.0	6.0
9-*cis*/*all-trans*-retinol	0.8	1.3	3.0	94.9

[a] The chemical esterification of the retinols and protocols for their HPLC analysis are described under Methods.

Analysis of [^{3}H]Retinoids Formed in Vitro. The hexane solution from the above incubation was mixed with carrier isomeric retinols and retinyl palmitates, and the isomers were separated as previously described in detail (Bernstein et al., 1987a). The isomeric retinols were separated on a 5-μm Merck Lichrosorb RT Si 60 silica column (250 × 4.0 mm) using a Waters high-performance liquid chromatography (HPLC) system. The retinols were detected at 320 nm. The eluant used was 7% dioxane in *n*-hexane at 2 mL/min, to provide optimum separation of 11-*cis*-retinol from 13-*cis*-retinol (Landers & Olson, 1984). The HPLC traces of the coinjected retinol standards were identical with previously published chromatograms (Fong et al., 1983). For radioactive analysis, the fractions were collected by using a Gilson 201 microprocessor-controlled fraction collector in the peak detection mode. Appropriate delay for dead volume between detector and dropping needle was programmed into the collector, and all base-line effluent was discarded. Fractions were counted in 4.5 mL of ScintiLene on a Beckman LS 1800 scintillation counter interfaced with an Apple-II$^+$ computer for data analysis. All counts were corrected for quench and background. In instances where the retinyl esters were analyzed, separations were performed with 0.5% diethyl ether in *n*-hexane as eluant (Bernstein et al., 1986). When the retinal isomers were analyzed, oximes were first formed, followed by methylene chloride extraction, and the *syn*-retinal oximes were separated with 8% diethyl ether in *n*-hexane as the eluant (Groenendijk et al., 1980a).

In order to be certain that the radioactivity collected in the peaks was as identified, the peak materials were collected, and a chemical structural analysis was performed. The experiment described here confirms that the isomeric retinols collected were authentic. The radioactive *all-trans*-retinol (2 μCi) was incubated with the membranes in the usual way, and the isomeric retinols were collected as above using 7% dioxane in *n*-hexane as eluant. It should be noted that *all-trans*-retinol and 9-*cis*-retinol coelute with this eluant. The collected retinols were separately esterified with palmitoyl chloride by standard means (Bridges & Alvarez, 1982) and rechromatographed to separate the isomeric esters using 0.5% diethyl ether in *n*-hexane. The results are given in Table I. Given that a small amount of isomerization will occur during the chemical esterification and chromatography procedures, these data establish the chemical identities of the collected retinol peaks beyond a doubt.

Subcellular Fractionation Procedures. The fractionation method used here was conducted according to a published

Table II: Cellular Location of the Bovine Isomerase[a]

cell homogenate	esterification[b] (% mg^{-1} min^{-1})	isomerization[c] (% mg^{-1} min^{-1})
retina	0.02	0.01
pigment epithelium	1.23	0.25

[a] The retinas from two fresh calf eyes were cleanly separated from the underlying pigment epithelium and homogenized in 0.1 M phosphate buffer using an ice-cold glass-on-glass homogenizer. The crude homogenate (1 mg) was then assayed for esterifying and isomerizing activity with 0.2 μCi of [^{3}H]-*all-trans*-retinol (specific activity 60 Ci/mmol) as described under Methods. Identical experiments were performed on the pigment epithelium membranes (750 g of supernatant). [b] Formation of [^{3}H]-*all-trans*-retinyl palmitate. [c] Formation of [^{3}H]-11-*cis*-retinol.

procedure (Feeney-Burns & Berman, 1982). The dissection, homogenization, fractionation, and enzyme marker assays were conducted on the same day to avoid complications due to freezing and thawing; 20 fresh bovine calf eyes were dissected with total retina removal. The retinal pigment epithelium cells were brushed out into the buffered sucrose medium and washed 4 times at 140 *g* in a swinging-bucket rotor. The cells were broken by nitrogen decompression at 5 °C and then fractionated by differential centrifugation according to the published protocol (Feeney-Burns & Berman, 1982).

Results

Formation of 11-cis-Retinoids by Bovine Pigment Epithelium Membranes as a Function of Time and Protein Concentrations. Initial experiments performed were aimed at demonstrating the existence of 11-*cis*-retinoid biosynthetic activity in the mammalian eye and determining its location. The first experiments reported here were aimed at determining the tissue site of the isomerizing activity. Two fresh calf eyes were dissected to produce retina and pigment epithelium tissue separately. Crude homogenates of the retina and a membrane preparation from the pigment epithelium were assayed for their abilities to produce 11-*cis*-retinol from added *all-trans*-retinol (Table II). Ester synthetase activity was also assayed. As seen in Table II, both 11-*cis*-retinol synthetase activity and ester synthetase activity are found almost exclusively in the pigment epithelium. Since both processes are endergonic in nature, an endogenous energy source must exist in these membranes. Attempts at detergent solubilization of these membranes, using a variety of detergents, have not been successful, as the activity was invariably irreversibly destroyed (B. S. Fulton and R. R. Rando, unpublished experiments).

In Figure 1A is shown a time course for 11-*cis*-retinol formation, using 50 μg of the standard pigment epithelium membrane preparation described under Methods. All further experiments were performed with similar membrane fractions from sonicated pigment epithelium membranes. As shown here, the amount of 11-*cis*-retinol produced leveled off after about an hour at this protein concentration, probably as a consequence of protein denaturation. In Figure 1B are shown data giving the synthesis of 11-*cis*-retinol as a function of protein concentration. Under the usual assay conditions, 10–15% of the total retinol was 11-cis at the end of the incubation period, and up to 60% of the *all-trans*-retinol added was directly esterified. The literature gives a value of 50–60% for the latter process under similar conditions (Berman et al., 1980). As determined by 13 different assays conducted as described above and under Methods, the mean isomerase activity with respect to the formation of 11-*cis*-retinol is 1.3 ± 0.1 pmol (mg of protein)$^{-1}$ min^{-1}. Of course, this is not a maximal velocity, since the K_M for the isomerization process cannot be determined without further purification. As will

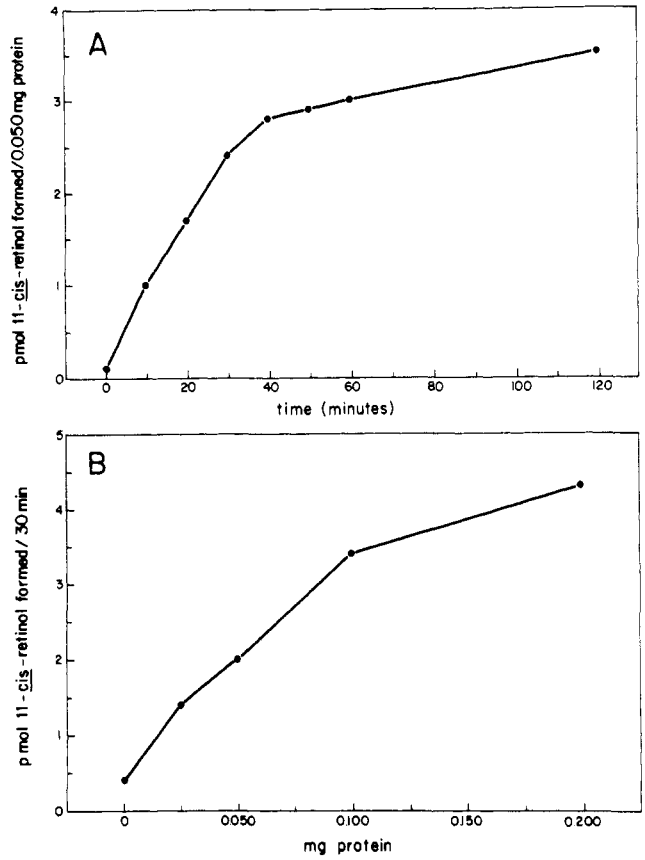


FIGURE 1: 11-*cis*-Retinol formation as a function of time and protein concentrations. In Figure 1A is shown a time course for the formation of 11-*cis*-retinol as a percentage of the total retinol pool when [^{3}H]-*all-trans*-retinol was incubated with 0.050 mg of the standard membrane preparation per milliliter total volume. Assays were performed as indicated under Methods. [^{3}H]-*all-trans*-Retinol at a concentration of 1 μM was used as substrate. In Figure 1B, similar assays were performed, varying the protein concentrations and incubating for a fixed period of time, in this case 30 min. Incubation of 1 μM *all-trans*-retinol with 0.2 mg of protein for 30 min produced 8.8% 11-*cis*-retinol and 70% of the isomeric retinyl palmitates.

be shown later, 11-*cis*-retinyl palmitate was also produced by these membranes.

Free all-trans-Retinol Is the Apparent Substrate for Isomerization. In the experiments reported above, *all-trans*-retinol was utilized as the substrate. However, given the metabolic complexity of the membranes used, it could not be assumed that the alcohol was the actual substrate for the isomerizing system. For example, it was already clear that retinyl esters are made by these membranes. Therefore, it was necessary to establish the actual substrate as far as possible in this system. As previously found in the amphibian system, 11-*cis*-retinyl palmitate, in addition to 11-*cis*-retinol, is synthesized by the membranes from added *all-trans*-retinol. It is noteworthy that 11-*cis*-retinal was not produced by the washed bovine pigment epithelium membranes, whereas substantial amounts were always found with the amphibian retina/pigment epithelium system (Bernstein et al., 1987a). In the following experiments, both the synthesis of 11-*cis*-retinyl palmitate and that of 11-*cis*-retinol were followed. In one instance, the possible synthesis of 11-*cis*-retinal was also followed. In Table III are shown data for the conversion of *all-trans*-retinol and *all-trans*-retinyl palmitate by the membrane fraction. As can be seen here, free *all-trans*-retinol was readily converted into 11-*cis*-retinol and 11-*cis*-retinyl palmitate. No measurable conversion occurred with *all-trans*-retinyl palmitate as the putative substrate.

Table III: Conversion of *all-trans*-Retinoid Substrates to 11-*cis*-Retinoids[a]

substrates	% of total retinols			% of total retinyl palmitates				% esters[b]
	11-cis	13-cis	all-trans	11-cis	13-cis	9-cis	all-trans	
all-trans-retinol	21	11	68	9	6.5	1	83.5	70
all-trans-retinyl palmitate		(1.6)[c]		0.5	0.6	0.4	98.5	98.4
all-trans-retinyl palmitate (control)		(0.7)[c]		0.6	1	0.5		99.3

[a] [^{3}H]-*all-trans*-Retinyl palmitate was synthesized from [^{3}H]-*all-trans*-retinol (specific activity 53 Ci/mmol) which had been diluted with cold *all-trans*-retinol. The [^{3}H]-*all-trans*-retinyl palmitate was HPLC purified to give approximately 6 μCi of pure [^{3}H]-*all-trans*-retinyl palmitate at a concentration of 0.03 μCi/μL. The assay was conducted with a total membrane preparation (0.15 mg of protein). 1 μCi of *all-trans*-retinyl palmitate (85 nM) was added, and the solution was incubated for 15 min at 38 °C. As a control, 0.2 μCi of *all-trans*-retinol was incubated under the same conditions. Controls (in the absence of eye tissue) were previously subtracted. [b] Gives the percent of esters in the total retinoid pool. [c] Refers to total retinols.

Table IV: Lack of Conversion of *all-trans*-Retinal to 11-*cis*-Retinoids[a]

substrates	% total retinols			% total retinyl palmitates				% total retinal[b] *syn*-oximes			% esters
	11-cis	13-cis	all-trans	11-cis	13-cis	9-cis	all-trans	11-cis	all-trans	13-cis	
all-trans-retinol	5	6	89	11	13.5	4.5	71		<1[b]		48
all-trans-retinal		<1[b]				<0.5[b]		0.4	88.8	10.8	<0.5

[a] [^{3}H]-*all-trans*-Retinal of specific activity 5 Ci/mmol and 0.1 μCi/μL was used here. The solution is approximately 20 μM in [^{3}H]-*all-trans*-retinal. The assay was conducted at 1 μM in *all-trans*-retinal and in a parallel experiment (1 μM *all-trans*-retinol was also assayed). All assays were in duplicate using 0.1 mg of protein with a 30-min incubation period. In this experiment, the retinal (via their *syn*-oximes), retinyl palmitate, and retinol isomeric compositions were determined. All data were corrected from controls. Assays and analyses were performed as described under Methods. [b] Refers to percent of total.

A second series of experiments was conducted comparing *all-trans*-retinol and *all-trans*-retinal as substrates (Table IV). It was clear here again that only free *all-trans*-retinol would serve as the substrate. Furthermore, 11-*cis*-retinal was not formed from *all-trans*-retinal nor was it formed from *all-trans*-retinol. This rules out the possibility that isomerization occurs with free aldehyde as the substrate, with further enzymatic reduction to the alcohol. Indeed, in these washed membranes, very little, if any, redox chemistry occurs.

Studies on the Subcellular Distribution of Isomerase Activity. As already shown above, isomerase activity was found almost exclusively in the pigment epithelium. It was of some interest to determine if further cellular fractionation of this organ would produce any useful information concerning the subcellular location of the isomerase. A procedure was designed to fractionate dissected pigment epithelium obtained from fresh calf eyes (Berman et al., 1980). This procedure was performed several times and did not reveal a specific locus for the isomerizing system. Succinate dehydrogenase was used as a marker enzyme for mitochondria and retinol ester synthetase for the microsomal fraction. It had previously been determined that the ester synthetase activity lies substantially, but by no means exclusively, in the microsomal fraction (Berman et al., 1980). As can be seen in Figure 2, the isomerase activity is distributed broadly through all of the membrane fractions. The material labeled by convention as cytoplasmic does not represent soluble isomerization activity, for it too can be centrifuged at higher speeds, for example, at 150000*g*.

The interpretation of these data is complicated by the fact that both the ester synthetase and the isomerase activity appear to use free *all-trans*-retinol as their substrate. Hence, a quantitative comparison of activities cannot be made, since the relative K_M's for the isomerase and the ester synthetase activities are unknown. Very high concentrations of *all-trans*-retinol could not be used because of its known detergent- and membrane-destabilizing properties (Dingle & Lucy, 1965). It is clear, however, that both activities are broadly distributed. A question of significant importance is whether they might also be functionally linked.

Chemical and Physical Probes of Isomerase and Ester Synthetase Activities. The sensitivity of the isomerase and

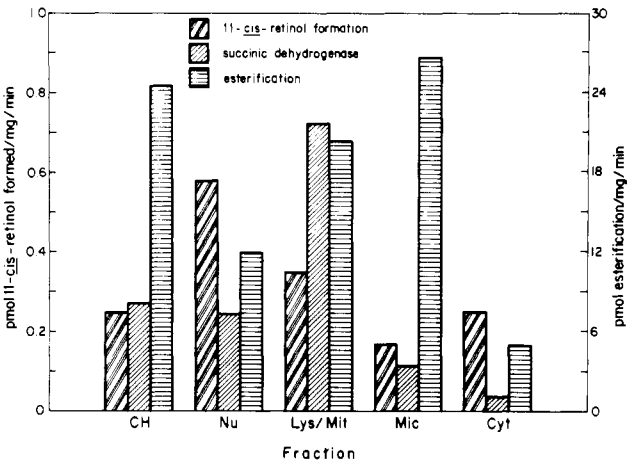


FIGURE 2: 11-*cis*-Retinol and *all-trans*-retinyl palmitate synthetase activities in subcellular fractions of the pigment epithelium. The subcellular fractionation was performed as indicated under Methods. Both *all-trans*-retinyl ester synthetase formation and 11-*cis*-retinol formation were assayed as also described under Methods. In the figure, CH = crude homogenate, Nu = nuclear fraction (750*g* pellet), Lys/Mit = lysosome/mitochondria (25000*g* pellet), and Cyt = cytosol (110000*g* supernatant). Isomerase assays were performed with 0.1 mg of protein.

ester synthetase activities to a variety of chemical agents was determined in order to help characterize the isomerization process. It was of interest to determine if these activities might be affected in a parallel manner by agents with widely disparate chemical and physical characteristics. Three different agents were chosen. Ethanol was chosen as a weak membrane-active agent, hydroxylamine because it can react with certain "high-energy" acyl bonds (Jencks, 1969), and *p*-(hydroxymercuri)benzoate as a sulfhydryl group modifying agent (Means & Feeney, 1971). In Figure 3 are shown data comparing the effects of ethanol on isomerase and ester synthetase activities in the standard membrane fraction. As can be seen here, both activities were sensitive to ethanol, but the isomerization activity was reduced by 93%, while the ester synthetase activity was reduced only by 67%.

In the experiments shown in Figure 4, the activities of the two processes are plotted as a function of the amount of *p*-(hydroxymercuri)benzoate, a reagent known to react with

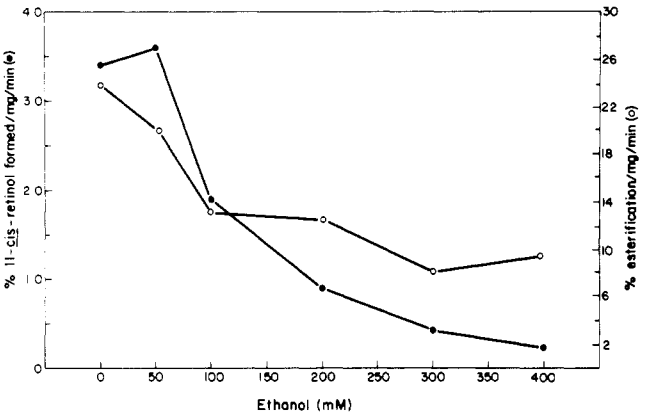


FIGURE 3: Effects of ethanol on 11-*cis*-retinol and *all-trans*-retinyl palmitate formation. The effects of ethanol at the indicated molarites (0.3–2.4% ethanol v/v) on the abilities of the standard membrane preparation to produce 11-*cis*-retinol and *all-trans*-retinyl palmitate were determined. All assays were performed as indicated under Methods.

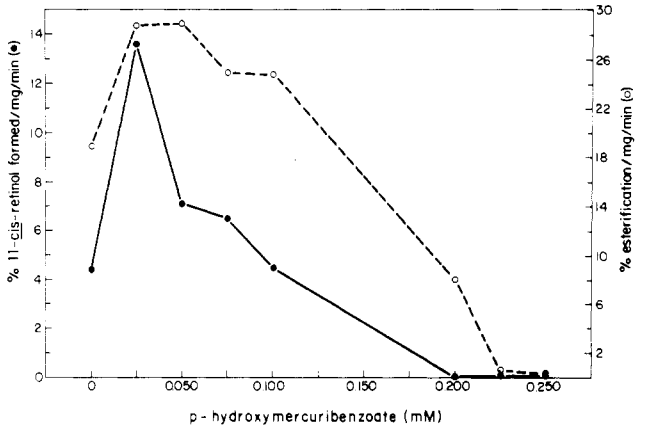


FIGURE 4: Effects of *p*-(hydroxymercuri)benzoate on 11-*cis*-retinol and *all-trans*-retinyl palmitate formation. The effects of *p*-(hydroxymercuri)benzoate at the indicated concentrations on the abilities of the standard membrane preparation to produce 11-*cis*-retinol and *all-trans*-retinyl palmitate were determined. All assays were performed as indicated under Methods.

sulfhydryl groups (Means & Feeney, 1971). Interestingly, at low *p*-(hydroxymercuri)benzoate concentrations, there was an actual stimulation of both activities, followed by a roughly parallel decrease at higher concentrations, suggesting some functional linkage between the two activities. This roughly parallel sensitivity was also manifested toward hydroxylamine (Figure 5). Although the ester synthetase has never been purified or characterized, it is known to be sensitive to hydroxylamine (Krinsky, 1958). Hydroxylamine readily cleaves thio esters and presumably inhibits ester synthetase, because it depletes the endogenous source of palmitoyl-CoA, or whatever other high-energy lipid ester substrate might be required in the formation of retinyl esters. Interestingly, as shown in Figure 5, hydroxylamine treatment also inhibited the isomerization process by an as yet undetermined mechanism.

DISCUSSION

In this paper, it is demonstrated that bovine eye contains an 11-*cis*-retinoid synthetase (isomerase) activity. This activity is heat and protease sensitive, as would be expected of a biological process in which enzymes are involved. Substantial amounts of 11-*cis*-retinol and its palmitate ester are produced by this activity from added *all-trans*-retinol. Both processes require the input of energy. Roughly equal amounts of the

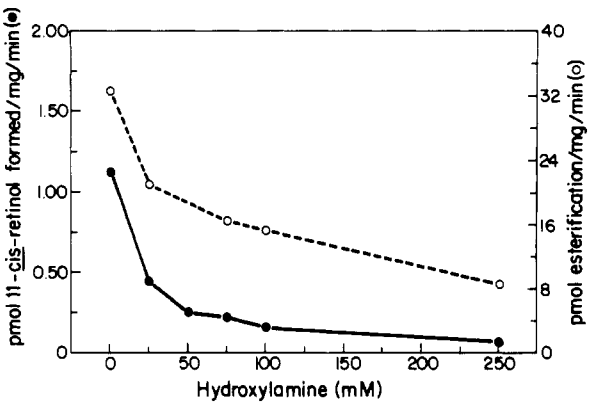


FIGURE 5: Effects of hydroxylamine on 11-*cis*-retinol and *all-trans*-retinyl palmitate formation. The standard membrane preparations were preincubated with hydroxylamine at the indicated concentrations at 38 °C for 30 min. After centrifugation and thorough washing, the abilities of the standard membrane preparation to produce 11-*cis*-retinol and *all-trans*-retinyl palmitate were determined. All assays were performed as indicated under Methods.

two 11-*cis*-retinoids are formed, which accounts for roughly 30–40% of the retinoids not converted to *all-trans*-retinyl palmitate. Since at chemical equilibrium 11-*cis*-retinoids account for only 0.1% of the equilibrium mixture, the aforementioned endergonic conversion must be biologically significant (Rando & Chang, 1983). The molecular interaction that makes 11-*cis*-retinoids unstable is an intramolecular one, so that the general milieu of the retinoids should not affect the position of equilibrium (Knowles & Dartnall, 1977b). This can be appreciated with nonspecific isomerization catalysts for the retinals, such as reduced flavin cofactors, phosphatidylethanolamine, rod outer segments and lipids derived therefrom (Futterman & Rollins, 1973; Groenendijk et al., 1980b). None of these catalysts produces any measurable 11-*cis*-retinal starting with either *all-trans*- or 13-*cis*-retinal (Futterman & Rollins, 1973; Groenendijk et al., 1980b).

The observation that there is an 11-*cis*-retinoid biosynthetic activity in the bovine ocular system complements and expands previously reported studies from the amphibian system, which for the first time documented the in vitro biosynthesis of 11-*cis*-retinoids (Bernstein et al., 1987a). There are several aspects of the bovine system which, taken together, make it uniquely useful with respect to studying the isomerization process. One is the size, which makes the quantitative dissection of the retina from the pigment epithelium possible and, in addition, allows for the potential large-scale preparation and purification of the isomerase.

The ability to cleanly dissect the retina from the pigment epithelium is important here, because the isomerase can now be localized as being entirely within the pigment epithelium, at least in mammals (Table II). This explains why pigment regeneration has never been observed to occur in excised retina preparations, even when exogenous retinoids are added (Pepperberg et al., 1978; Pepperberg & Masland, 1978). A further useful property of the bovine system is the relative lack of redox activity found in the washed membranes. An abundance of endogenous redox activity complicated analysis in the amphibian, because even washed membranes oxidized retinol and reduced retinal, making it difficult to be absolutely certain about the oxidation state of the isomerized substrate (Bernstein et al., 1987a). In the washed membranes from the bovine system described here, there was no measurable redox chemistry, allowing the unambiguous conclusions to be drawn that the isomerization substrate is at the alcohol level of oxidation (Tables III and IV). This result is consistent with in

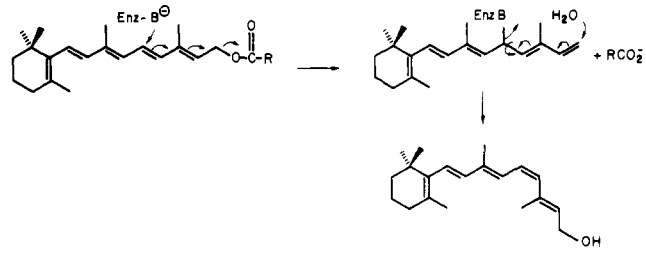
vivo results from the rat using a double labeling technique, and with in vitro studies on the frog pigment epithelium membranes (Bernstein & Rando, 1986; Bernstein et al., 1987a). Finally, application of the double labeling technique, which follows the fate of the ^3H of the substrate [15-^{14}C,15-^3H]-*all-trans*-retinol to the bovine system described here, showed no loss of ^3H during isomerization (Bernstein et al., 1987b).

Interestingly, the enhanced in vitro redox activity observed in the amphibian versus the bovine system appears to parallel that found in vivo. Known potent inhibitors of alcohol dehydrogenases markedly decrease the rate of pigment regeneration during dark adaptation in mammals, but not in amphibians, suggesting an overabundance of alcohol dehydrogenase activity in the latter species (Bernstein & Rando, 1986). In vivo double labeling experiments employing [15-^{14}C,15-^3H]-*all-trans*-retinol showed a markedly more rapid alcohol dehydrogenase dependent tritium washout in amphibians compared to mammals (Bernstein & Rando, 1986).

Although it was possible to determine that the isomerase is localized to the pigment epithelium, subcellular localization at a higher resolution was not possible. It did not appear to us, using standard membrane isolation procedures, that the isomerizing activity resided in a particular membrane fraction (Figure 2). It appears that the isomerase is present in all membrane fractions and not localized to a particular organelle. The method of processing the membranes preparatory to carrying out the studies described in Figure 2 involved the gentle opening of the cells so as not to destroy a particular organelle should it exist. This treatment left a substantial amount of isomerase activity in large membrane fractions—the so-called nuclear fraction. We believe it highly unlikely that substantial isomerization activity resides in the nucleus. Rather, it is probable that the enzyme(s) involved in the isomerization process is (are) nonspecifically localized in intracellular membranes. The same conclusion was arrived at by density gradient centrifugation studies on the amphibian system, where the isomerase activity was found to be broadly distributed across the various gradients (Bernstein et al., 1987b). A further difficulty in attempting to establish a membrane location for the isomerase activity is that even in the washed membranes, the added substrate *all-trans*-retinol is processed both by the isomerase and by the ester synthetase. If the two activities are unlinked, then the substrate must be partitioned by the two processes according to their respective binding constants and catalytic efficiencies, the relative values of which are unknown. Thus, the relatively small amount of isomerization activity found in the microsomal fraction, where ester synthetase is known to be high, may be a reflection of the rapid esterification of the available *all-trans*-retinol by the ester synthetase (Berman et al., 1980). In the crude membrane fraction, approximately 60% of the added *all-trans*-retinol was converted into *all-trans*-retinyl palmitate.

The experiments described above make it difficult to prove or disprove that ester synthetase and the isomerase copurify. Whether or not the isomerase is a multicomponent system is very important with respect to deciding on the proper approach to its eventual purification and characterization. For example, we have not been able to find a detergent capable of solubilizing the system without apparently denaturing it. Interestingly, similar experiences have been reported with the ester synthetase (Krinsky, 1958). If the isomerase is multicomponential in nature, then this would be expected, because detergents could easily destroy critical interactions between the components of the system. In fact, there is a reason why a multicomponent system might be expected. The reason is that the isomerizing system must drive a thermodynamically uphill process. This isomerizing system is unlike any known isomerase reported, for that reason alone. It must probably utilize metabolic energy to accomplish its task, as it is unlikely that the one binding protein which specifically recognizes 11-*cis*-retinal(ol) is found in high enough concentrations to be relevant, since the binding protein would have to be used stoichiometrically and not catalytically (Saari & Bredberg, 1982).

Scheme I



One possible mechanism for coupling metabolic energy to the isomerization is shown in Scheme I. (Scheme I shows a fatty acid ester, but other esters could provide the necessary energy by the same mechanism.) In this mechanism, the free energy of ester hydrolysis is used to drive the isomerization. The free energy of the hydrolysis of esters can be in the −5 to −10 kcal/mol range, which is enough to make up the 4 kcal/mol difference between 11-*cis*- and *all-trans*-retinoids (Rando & Chang, 1983; Jencks, 1970).[1] However, on the surface, this hypothesis might be considered disproved, because *all-trans*-retinyl palmitate, the major retinyl ester produced by this system, is not converted to 11-*cis*-retinoids by the isomerase reported here, nor by the amphibian system (Bernstein et al., 1987a). It must be remembered, though, that the retinyl palmitates are exceedingly hydrophobic compounds and do not partition well into the aqueous phase, which they would have to do in order for retinoid exchange and transfer to occur. For example, retinol and retinal undergo intermembranous transfer at very rapid rates, with half-lives in seconds (Rando & Bangerter, 1982). Under the same conditions, the retinyl palmitates are not exchanged, even after hours (Rando & Bangerter, 1982). Thus, it is in the realm of possibility that the *all-trans*-retinyl palmitate added in the experiments described simply does not enter the membrane pool accessible to the isomerase. Of great interest, along these lines, is the very recent article on retinyl palmitate esterases from the pigment epithelium (Blaner et al., 1987). It was reported that *all-trans*-retinyl palmitate was only a substrate for the esterase when it was added in detergent. This strategy is, of course, not possible here because of the great sensitivity of the isomerization activity to detergents.

A study of the sensitivity of the isomerases and ester synthetase to various chemical agents was undertaken, both to characterize the properties of the isomerase and to determine if there is a possible linkage between the isomerase system and the ester synthetase. The effects of ethanol on both systems were similar, although not identical (Figure 3). Ethanol had a very profound irreversible denaturing effect on the isomerase. At ethanol concentrations above 2%, less than 10% of the activity remained. This might explain why the isomerase had been missed before in in vitro systems, because it had been customary to add the retinoid substrates in 2–4% ethanol (Fong et al., 1983; Flood et al., 1983). The denaturing effect

[1] The acutal values for retinyl esters are unknown but will clearly depend on the nature of their microenvironment.

of ethanol on the ester synthetase proved to be less profound, because even at 2.4% ethanol approximately 38% of the activity remained. The effect of hydroxylamine on the isomerizing and ester synthetase activities was also studied (Figure 5). The rationale behind these experiments was as follows. Hydroxylamine had already been shown to inhibit ester synthetase, possibly by a mechanism which involves the cleavage of a high-energy acyl donor (Krinsky, 1958). Recent evidence suggests that this enzyme can be stimulated by an acyl-CoA (Saari & Bredberg, 1987). This inhibition is recapitulated here in Figure 5. The hydroxylamine decreased the ester synthetase activity by about 75% at 250 mM concentration. No additional inhibition was observed at a hydroxylamine concentration of 500 mM. Hydroxylamine also had a profound effect on the isomerase, reducing it to 5% at a concentration of 250 mM (Figure 5). This might suggest that there is some linkage between the ability of these membranes to make retinyl esters and their ability to carry out the isomerization process. It should be remembered that both processes are endergonic in nature, so that it is possible and perhaps even likely that the hydroxylamine is not intercepting a high-energy acyl intermediate but is simply preventing energy utilization at an earlier step by some as yet unknown mechanism. The question remains, however, as to why hydroxylamine should inhibit both isomerization and esterification if these processes are not in some way linked.

This possible linkage is made more probable by the results of the experiments with *p*-(hydroxymercuri)benzoate, a reagent known to react irreversibly with sulfhydryl groups (Figure 4) (Means & Feeney, 1971). The effect of this reagent on both ester synthesis and 11-*cis*-retinol synthesis was biphasic (Figure 4). At low concentrations of the reagent, both activities were substantially enhanced, although a more pronounced effect was noted on the synthesis of 11-*cis*-retinol. The enhancement of both processes found a maximum at approximately 25 μM and decreased in a parallel fashion thereafter. The enhancement of enzymatic activities in the presence of low concentrations of *p*-(hydroxymercuri)benzoate has been observed before and has been shown in many cases to be due to the dissociation of an enzyme's catalytic and regulatory subunits, releasing the enzyme from inhibition (Means & Feeney, 1971). Interestingly, the decrease in both activities at higher inhibitor concentrations found a plateau between approximately 50 and 100 μM *p*-(hydroxymercuri)benzoate before decreasing further. The isomerization and esterifying activities were completely inhibited at 200 and 225 μM *p*-(hydroxymercuri)benzoate, respectively. Other sulfhydryl-reactive molecules were also found to abolish both activities. For example, 10 mM sodium iodoacetate decreased the isomerase by 91% and the esterification activity by 88%, and 50 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) completely inhibited both activities.

The results discussed above certainly suggest that the isomerization and esterification activities are importantly related. The reagents discussed all inhibited both activities in roughly parallel fashion, although, in general, the isomerase proved to be somewhat the more sensitive of the two. Of course, it is always possible that the parallelism in sensitivities observed is fortuitous and that the activities are not related. We believe that this is unlikely, given that the reagents chosen were unrelated in their biological actions. Furthermore, it would be difficult to comprehend how hydroxylamine might inhibit the isomerase and why both activities should be stimulated at low *p*-(hydroxymercuri)benzoate concentrations but inhibited at high concentrations, if some linkage between the two processes were not obligate. The nature of this possible linkage is the subject of current study.

Registry No. *all-trans*-Retinol, 68-26-8; 11-*cis*-retinol, 22737-96-8; 11-*cis*-retinyl palmitate, 51249-33-3; *all-trans*-retinyl palmitate, 79-81-2; ethanol, 64-17-5; hydroxylamine, 7803-49-8; *p*-(hydroxymercuri)benzoate, 1126-48-3; ester synthetase, 74665-81-9; isomerase, 109740-80-9.

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Action of Lecithin–Cholesterol Acyltransferase on Low-Density Lipoproteins in Native Pig Plasma†

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ABSTRACT: The action of lecithin–cholesterol acyltransferase (LCAT, EC 2.3.1.43) on the different pig lipoprotein classes was investigated with emphasis on low-density lipoproteins (LDL). It was demonstrated previously that LDL can serve as substrate for LCAT, probably because they contain sufficient amounts of apoA-I and other non-apoB proteins, known as LCAT activators. Upon a 24-h incubation of pig plasma in vitro in the presence of active LCAT, both pig LDL subclasses, LDL-1 and LDL-2, fused together, forming one fraction, as revealed by analytical ultracentrifugation. This fusion was time dependent, becoming visible after 3 h and complete after 18 h of incubation. Concomitantly, free cholesterol and phospholipids decreased and cholesteryl esters increased. When isolated LDL-1 and LDL-2 were incubated with purified pig LCAT for 24 h, LDL-1 floated toward higher densities and LDL-2 toward lower densities, although this effect was not as pronounced as in incubations of whole serum. In further experiments, pig serum was incubated for various periods of time in the presence and absence of the LCAT inhibitor sodium iodoacetate. The individual lipoproteins then were separated by density gradient ultracentrifugation or by specific immunoprecipitation and chemically analyzed. Both methods revealed that in the absence of active LCAT there was a transfer of free cholesterol from LDL to high-density lipoproteins (HDL) and a small transfer of cholesteryl esters in the opposite direction. In the presence of LCAT the loss of free cholesterol started immediately in all three lipoprotein classes, was most prominent in LDL, and was proportional to the newly synthesized cholesteryl esters incorporated in each fraction. In contrast, the loss of phospholipids was not proportional to the newly formed cholesteryl esters in each lipoprotein class: whereas very low density lipoproteins and LDL lost smaller amounts of phospholipids, HDL lost more phospholipids than necessary for the synthesis of cholesteryl esters incorporated in each class. At any time the greatest amount (60–70%) of newly synthesized cholesteryl ester was found in LDL. From these results it is concluded that in pig serum LCAT acts significantly—if not preferentially—on apoB-containing lipoproteins, even in the presence of physiological concentrations of HDL.

Lecithin–cholesterol acyltransferase (LCAT,[1] EC 2.3.1.43) is responsible for the major portion of cholesteryl esters circulating in plasma (Glomset, 1973, 1979). Recently, the catalytic mechanism of this enzyme from human plasma was elucidated (Jauhiainen & Dolphin, 1986). High-density lipoproteins (HDL) are believed to serve as the main substrate for human LCAT, and apoA-I was found to be a necessary cofactor for the LCAT reaction (Fielding et al., 1972). However, it was shown that besides apoA-I other apolipoproteins, e.g., apoC-I, apoE, and apoA-IV as well as apoA-II, are also able to activate human LCAT to a certain extent (Soutar et al., 1975; Albers et al., 1979; Steinmetz & Utermann, 1984; Zorich et al., 1985; Chen & Albers, 1986). Additionally, it was shown that crude LCAT preparations derived from pig plasma and also purified LCAT derived from either human or pig plasma interact directly with very low density lipoproteins (VLDL) and low-density lipoproteins (LDL) (Barter, 1983; Barter & Hopkins, 1983; Knipping et al., 1986). Finally, it was reported that together with triglyceride-rich particles and cholesteryl ester transfer protein (CETP) LCAT plays a major role in the interconversion of HDL-3 to HDL-2 (Dieplinger et al., 1985; Knipping et al., 1985).

This paper is devoted to the elucidation of the direct action of LCAT on LDL in native serum, which seems to be of particular relevance, as it was recently published that in patients having fisheye disease LCAT acts only on combined VLDL and LDL and not on HDL (Carlson & Holmquist, 1985a,b). In this disease there is a low relative content of cholesteryl esters in HDL, but a normal content of these lipids in VLDL and LDL. Additionally, HDL of fisheye disease plasma are smaller than normal. However, they are good substrates if lipoprotein-deficient plasma of normal subjects is used as the source of LCAT.

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[1] Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; pig LDL-1, buoyant density fraction 1.020–1.063 g/mL; pig LDL-2, buoyant density fraction 1.063–1.080 g/mL; VLDL, very low density lipoproteins; LCAT, lecithin–cholesterol acyltransferase; CETP, cholesteryl ester transfer/exchange protein.

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