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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXVII. STEREOSPECIFICITY OF OCULAR RETINOL DEHYDROGENASES AND THE VISUAL CYCLE

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Summary

A comparative study is made of the stereospecificity of two particulate retinol dehydrogenases from bovine eyes and of horse liver alcohol dehydrogenase. The particulate retinol dehydrogenase of outer segments reacts with the all-*trans* isomers of retinaldehyde and retinol but not with the 11-*cis* compounds. In contrast, a particulate retinol dehydrogenase present in pigment epithelium reacts preferentially with the 11-*cis* compounds. Horse liver alcohol dehydrogenase (EC 1.1.1.1) can convert both isomers, but the all-*trans* isomers are clearly preferred. Differences with regard to cofactor preference and stability are also noted.

The outer segment enzyme clearly functions in the rhodopsin cycle. It is unlikely that the 11-*cis* retinol dehydrogenase from pigment epithelium is directly involved in providing 11-*cis* retinaldehyde for rhodopsin regeneration, but it may serve to make available 11-*cis* retinaldehyde from rhodopsin, digested in phagocytized rod sacs, for the synthesis of visual pigment by the visual cells.

Introduction

Oxidoreductases play an important role in the visual cycle [1—5]. Recently we have shown that the retinol dehydrogenase activity of cattle rod outer segments is stereospecific. It readily converts all-*trans* retinaldehyde into all-*trans* retinol and vice versa, but 11-*cis* compounds only after previous isomerization [6]. Subsequently, we have noticed the presence of a different retinol dehydrogenase in the pigment epithelium. In this study we describe some properties of this enzyme, and compare it to other oxidoreductases from the retina and the liver, which react with retinaldehyde and retinol.

Materials and Methods

Substrates

All-*trans* retinaldehyde is obtained from Eastman, Rochester, N.Y.; 11-*cis* retinaldehyde is prepared by photo-isomerization of an all-*trans* retinaldehyde solution in ethanol and elution of the 11-*cis* isomer on an aluminum oxide column with benzene/hexane (1 : 9 v/v)[7]. All-*trans* retinol and 11-*cis* retinol are prepared by reducing the corresponding retinaldehyde with $NaBH_4$ in ethanol, followed by extraction into hexane. Experiments involving the vitamin A compounds* are carried out in dim red light to prevent light-induced isomerization, and under N_2 to prevent oxidation.

Enzyme sources

Unless otherwise indicated outer segment preparations are isolated by the method of de Grip et al. [8] in daylight. After the first sucrose gradient centrifugation of this procedure, a heavy sediment and two layers are obtained. The upper layer, which contains exclusively rod outer segments, is collected with care to prevent contamination with the lower layer, diluted with an equal volume of Tris · HCl buffer (0.16 M, pH 7.1) and centrifuged at 27 000 × *g* and 4° C for 15 min. The sediment is washed twice with distilled water and sedimented after each washing (45 000 × *g*, 4° C, 30 min). After resuspension in an appropriate buffer the material is used as the enzyme preparation. During the normal isolation procedure in daylight all rhodopsin is bleached and the resulting preparation does not contain detectable amounts of either retinaldehyde or retinol. When the isolation procedure is carried out in dim red light, the upper layer contains all the rhodopsin.

Pigment epithelium is isolated by gently scraping it off the choroidal tissue of the same bovine eyes from which the retinas have previously been removed. This material is homogenized in ice-cold Tris · HCl buffer (0.16 M, pH 7.1) and centrifuged at 500 × *g* for 5 min. The supernatant is then centrifuged at 43 000 × *g* for 1 h. The resulting sediment is washed twice with distilled water. After resuspending it in the appropriate buffer (see below), the material is used as the enzyme preparation. The absence of rod outer segment material is assured by the absence of both rhodopsin and opsin when the procedure is carried out in darkness.

Horse liver alcohol dehydrogenase, which is purchased from Boehringer (Mannheim, Germany), contains 10% ethanol added as a preservative. Since ethanol interferes with the conversion of vitamin A compounds by this enzyme, the enzyme preparation is dialyzed for 3 h against the appropriate buffer (see below) shortly before it is used in an experiment.

Methods

Oxidoreductase activity is determined by measuring the velocity of substrate conversion during the first 3 min of incubation. Both in the reduction and the oxidation reactions the amount of retinaldehyde present is measured as a function of incubation time, showing the disappearance of the substrate or

* The term "Vitamin A compounds" in this paper includes retinol, retinaldehyde and retinylesters.

the appearance of the product. Unless otherwise indicated, the reaction is carried out in 0.5 ml detergent-free medium, containing an amount of enzyme sufficient to give a maximal conversion rate of 20 nmol per 5 min at 37°C. The reduction reaction required about 50 μg of pigment epithelium preparation and 350 μg of rod outer segment preparation, while for the oxidation reaction, 5—15 times more protein is necessary. The substrate (60 nmol) is added in 10 μl methanol. The presence of this small amount of organic solvent does not affect the enzyme activity. The reduction reaction is carried out in 0.1 M sodium acetate buffer (pH 5.0) and the oxidation reaction is 0.1 M Tris · HCl buffer (pH 8.5). The reaction is started by adding 300 nmol of the appropriate cofactor and at definite intervals aliquots are taken, frozen in dry ice/ethanol and later analyzed for their retinaldehyde content by the thiobarbituric acid method [9,10].

Product analysis is carried out by thin-layer chromatography. The samples are repeatedly extracted with a double volume of hexane. The concentrated hexane solutions are applied to Silicagel 60 F 254 plates (Merck, Darmstadt, Germany) and eluted with 50% (v/v) ether/hexane in saturated chambers. The developed chromatograms are scanned with a Vitatron TLD-100 densitometer by measuring the absorption of transmitted light from a tungsten source provided with a filter which transmits light of wavelengths between 250 and 400 nm.

Enzymatic formation of 11-*cis* retinaldehyde is also tested by its reaction with opsin. The opsin preparation is the upper layer of the retina fractionation carried out in the light. The amount of rhodopsin formed during this reaction is determined by differential spectroscopy in 1% Triton X-100 in 0.1 M phosphate buffer (pH 7.0) containing 50 mM hydroxylamine. Further details are given in Table III and Fig. 4.

Results

Assay conditions

The ocular oxidoreductases which are able to convert vitamin A compounds seem to be largely membrane-bound, since after centrifugation of reti-

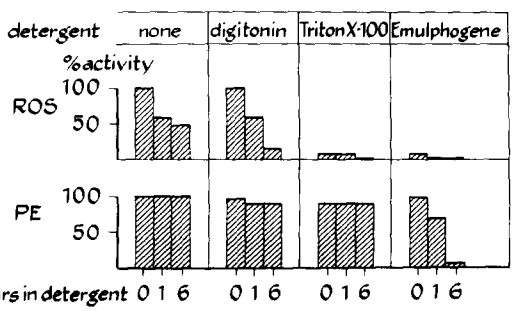

Fig. 1. Effect of detergents on the activity of retinol dehydrogenases from outer segments (ROS) and from pigment epithelium (PE). Initial rate of retinaldehyde conversion is measured in 0.5 ml of a fresh enzyme suspension in 0.1 M acetate buffer pH 5.0 and in similar mixtures containing 1% (w/v) detergent, by incubation at 37°C with 60 nmol substrate in the presence of 300 nmol of the appropriate coenzyme. The assay is repeated after 1 and 6 h during which the enzyme is kept at 20°C.

nal homogenates at 100 000 × *g* for 10 min the resulting supernatant is completely devoid of such enzyme activity [5]. Detergents have often been used in studies of these enzymes, in view of their particulate nature and of the water insolubility of vitamin A compounds. However, the activity of ocular oxidoreductases is seriously affected by detergents (Fig. 1). Therefore, we have used suspensions of particulate material and have added the substrate in a minimal amount of organic solvent. Unfortunately, liver alcohol dehydrogenase shows no activity at all towards retinaldehyde and retinol unless a detergent or membrane-material, e.g. enzymatically inactive opsin, is added. Therefore, all experiments with liver alcohol dehydrogenase have been performed in 0.1% Triton X-100, in which the enzyme is stable and active.

The effective substrate concentration of vitamin A compounds in a membrane suspension is difficult to assess, since the substrate accumulates in the lipid phase of the membranes. This precludes a realistic interpretation of binding and catalytic properties in terms of K_m and V values. The results of our experiments are therefore presented on a strictly comparative basis for substrate conversion rates of about 20 nmol per 5 min at 37° C.

Optimal pH and cofactor specificity

The optimal pH and the most efficient cofactor are determined for both retinaldehyde reduction and retinol oxidation. Initial velocities are measured using the most active stereoisomer for the enzyme under investigation (see below). For all three enzymes tested, the pH optimum for retinaldehyde reduction is at pH 5.0 and for retinol oxidation at pH 8.5. The results on cofactor preference are presented in Table I. The outer segment enzyme shows a higher activity with NADPH and $NADP^+$ than with NADH and NAD^+, the pigment epithelium enzyme does not show a clear preference and liver alcohol dehydrogenase works more efficiently with NADH and NAD^+. Approximately the same ratios of cofactor efficiency are obtained at pH 7 as at the optimal pH.

Stereospecificity

The conversion rates of the all-*trans* and 11-*cis* vitamin A compounds have been compared under the optimal conditions described before. As shown in Table II and Fig. 2, the outer segment enzyme oxidizes and reduces all-*trans* compounds more rapidly than it does 11-*cis* compounds. The same pattern is

TABLE I

CO-ENZYME PREFERENCE OF OXIDOREDUCTASES

The relative co-enzyme preference is expressed as the ratio of the initial velocity of the reaction with NADP(H) to that with NAD(H), using as substrate the most active geometrical isomer for each enzyme.

Oxidoreductase	Substrate configuration	Ratio initial velocities with NADP(H) vs. NAD(H)	
		Reduction	Oxidation
Rod outer segment retinol dehydrogenase	all-*trans*	1.5	3.0
Pigment epithelium retinol dehydrogenase	11-*cis*	1.0	0.6
Liver alcohol dehydrogenase	all-*trans*	0.6	0.5

TABLE II

STEREOSPECIFICITY OF THREE OXIDOREDUCTASES

The relative stereospecificity of these enzymes towards 11-*cis* and all-*trans* retinaldehyde and retinol is expressed as the ratio of initial velocities of the reactions with each of the two isomers.

Oxidoreductase	Relative stereospecificity $\frac{\text{11-}cis}{\text{all-}trans}$	
	Reduction (pH 5.0)	Oxidation (pH 8.5)
Rod outer segment retinol dehydrogenase	0.1	0.4
Pigment epithelium retinol dehydrogenase	1.4	10.1
Liver alcohol dehydrogenase	0.2	0.3

shown by liver alcohol dehydrogenase, but the enzyme from pigment epithelium shows the reverse behaviour: here 11-*cis* compounds are the preferred substrates. Similar results are obtained, when the enzymes are tested at pH 7.

A serious problem in the interpretation of the initial conversion rates arises from the non-specific isomerisation of 11-*cis* vitamin A compounds. Under a variety of conditions, 11-*cis* compounds are isomerized even in darkness to a mixture of geometrical isomers [6,11]. Therefore, it is necessary to analyse the geometry of the product of enzymatic conversion. Thin-layer chromatography permits separation of the various isomers of retinaldehyde and retinol. Fig. 3 shows a typical experiment, in which 11-*cis* retinol is oxidized by the outer segment enzyme and the pigment epithelial enzyme, respectively. Although the separation between 11-*cis* and 13-*cis* vitamin A compounds is not complete, the same patterns are found in a large number of experiments. This pattern clearly indicates that the outer segment enzyme does not produce 11-*cis* retinaldehyde from 11-*cis* retinol, but that the pigment epithelial enzyme does [8].

Even stronger evidence for this conclusion is presented by the experiments summarized in Table III, in which the formation of 11-*cis* retinaldehyde during the enzymatic oxidation reaction is followed by its reaction with opsin to form rhodopsin. When bleached rod outer segments (the upper layer, containing both retinol dehydrogenase and opsin) are incubated with 11-*cis* retinol and $NADP^+$ or NAD^+ between pH 6.5 and 8.5, no rhodopsin is formed. When either

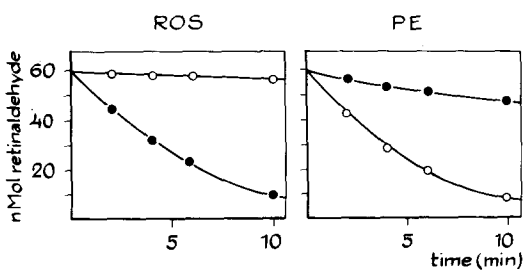

Fig. 2. Stereospecificity of retinol dehydrogenases from rod outer segments (ROS) and from pigment epithelium (PE) in the reduction of retinaldehyde. Activity is measured by incubating 60 nmol substrate (all-*trans* retinaldehyde, closed circles; 11-*cis* retinaldehyde, open circles) with a fresh suspension of the enzyme in 0.1 M acetate buffer (pH 5.0) at 37°C. 300 nmol of NADPH is added to the outer segment enzyme suspension and 300 nmol of NADH to the pigment epithelium enzyme suspension.

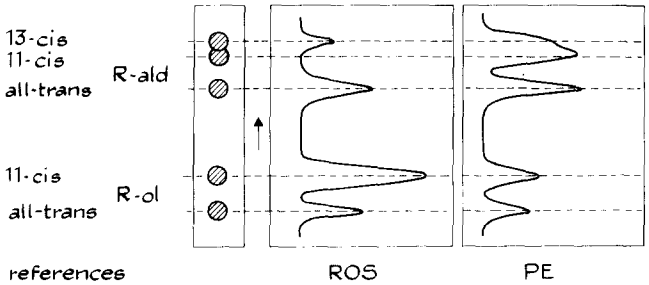

Fig. 3. Thin-layer chromatographic analysis of the products formed by oxidation of 11-*cis* retinol by retinol dehydrogenase from rod outer segments (ROS) and pigment epithelium (PE). The left part of the figure shows the positions of retinaldehyde (R-ald) and retinol (R-ol) isomers on the developed chromatogram and the right part shows densitometric scans of developed chromatograms containing the products of enzymatic oxidation of 11-*cis* retinol. Freshly prepared enzyme suspended in 0.5 ml 0.1 M Tris · HCl buffer pH 8.5, is incubated with 50 nmol 11-*cis* retinol for 15 min at 37°C, in the presence of 250 nmol $NAD(P)^+$. Vitamin A compounds are extracted with hexane and applied to silicagel thin-layer chromatography plates, which are developed with 50% (v/v) ether/hexane and scanned with a Vitatron TLD-100 densitometer.

TABLE III

ENZYMATIC OXIDATION OF 11-*cis* RETINOL

Enzymatic oxidation of 11-*cis* retinol to 11-*cis* retinaldehyde was measured by its ability to form rhodopsin. For conditions, see legend to Fig. 4. The opsin preparation also contains a retinol dehydrogenase specific for all-*trans* retinol.

Incubation mixture	$\Delta A_{500\ nm}$
Opsin + 11-*cis* retinol + $NAD(P)^+$	0.040
+ 11-*cis* retinol + NAD^+ + pigment epithelium retinol dehydrogenase	0.460
+ NAD^+ + pigment epithelium retinol dehydrogenase	0.020
+ 11-*cis* retinol + NAD^+ + liver alcohol dehydrogenase	0.460
Control: opsin + 11-*cis* retinaldehyde	0.440

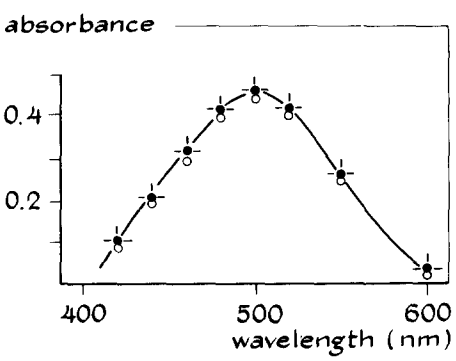

Fig. 4. Enzymatic oxidation of 11-*cis* retinol to 11-*cis* retinaldehyde measured by its ability to form rhodopsin. 15 nmol cattle opsin is incubated for 30 min in the dark at 37°C with 75 nmol 11-*cis* retinol and 350 nmol NAD in the presence of pigment epithelium enzyme (●) or liver alcohol dehydrogenase (-|-) in a total volume of 1 ml 0.1 M phosphate buffer, pH 7.0. In a control experiment (○) the same amount of opsin is incubated with 40 nmol 11-*cis* retinaldehyde. The amount of rhodopsin formed is determined by differential spectroscopy before and after illumination in the presence of 50 mM hydroxylamine after addition of Triton X-100 to a final concentration of 1%.

the pigment epithelium enzyme or liver alcohol dehydrogenase are added to this system all the opsin is converted to rhodopsin (Fig. 4). Formation of 11-*cis* retinaldehyde from all-*trans* retinol, or of 9-*cis* retinaldehyde from either 11-*cis* or all-*trans* retinol has never been observed [8].

Discussion

Vitamin A compounds are essential components of the visual process. In vertebrate eyes they occur predominantly in the retina and the pigment epithelium. In addition to retinaldehyde and retinol, retinyl esters are involved as a storage form [4,12]. Oxidoreductases, which catalyse the conversion between retinaldehyde and retinol, must play a role in the functional relationship between these different compounds. This role is made more complex by the occurrence in the eye of two geometric configurations of the vitamin A compounds, 11-*cis* and all-*trans*. Therefore, a careful characterization of the properties of these enzymes is necessary.

We find in the eye at least two different particulate oxidoreductases. One, which is tightly bound to rod outer segment membranes, has been described [2,3,5]. However, the one which occurs in the pigment epithelium has not been characterized before, though histochemical studies of the rat eye suggest the occurrence of an oxidoreductase in the pigment epithelium, which is different from the oxidoreductase present in the rod outer segments [13]. The strongest reason to believe that we are dealing with two different enzymes is their different stereospecificity towards 11-*cis* and all-*trans* vitamin A compounds. In addition, the two enzymes show a somewhat different cofactor preference and differ greatly in stability, for the enzyme from pigment epithelium has a greater thermal stability and is more resistant against inactivation by detergents (see Fig. 1). A more precise localization of the enzymes within the pigment epithelium and outer segments is currently being attempted by cell fractionation procedures and the use of marker enzymes. Both the outer segment and pigment epithelium enzymes differ from liver alcohol dehydrogenase in their particulate character, in coenzyme preference (Table I) and in stereospecificity (Table II).

What may be the functions of two different vitamin A-converting oxidoreductases in the retina and pigment epithelium, especially in view of their different stereospecificity? The rod outer segment enzyme is generally thought to be involved in the rhodopsin cycle [4]. All-*trans* retinaldehyde from photolyzed rhodopsin is reduced to all-*trans* retinol, most of which leaves the outer segment and is esterified in the pigment epithelium [14,15]. During regeneration of rhodopsin the reverse process occurs. Somewhere reisomerization to the 11-*cis* configuration must take place, since 11-*cis* retinaldehyde spontaneously combines with opsin to rhodopsin. However, it is still a matter of dispute at which step in the cycle (retinyl ester, retinol, retinaldehyde) and at which location (pigment epithelium, outer segments) the isomerization occurs.

From what we presently know about the metabolism of vitamin A compounds in the eye, the immediate precursor of 11-*cis* retinaldehyde, which is essential for visual pigment formation, could be either 11-*cis* retinol (through a dehydrogenase reaction) or all-*trans* retinaldehyde (through as isomerase reac-

tion). It is unlikely that 11-*cis* retinyl ester is a direct precursor, since enzymes catalyzing the conversion of esters to aldehydes are unknown.

If 11-*cis* retinaldehyde were formed outside the outer segments, either from 11-*cis* retinol or from all-*trans* retinaldehyde, it would have to migrate to the outer segment. However, transport of free retinaldehyde has never been observed in the eye [14,15] or elsewhere in the body, and is unlikely in view of the reactivity of the aldehyde group. Moreover, free 11-*cis* retinaldehyde is susceptible to nonspecific (i.e. light and enzyme-independent) isomerization to other geometric configurations [6,11]. Thus, in spite of the fact that the pigment epithelium contains enzymes able to catalyze the conversion of 11-*cis* retinyl esters, via 11-*cis* retinol [16], to 11-*cis* retinaldehyde, it is improbable that the pigment epithelium acts as a source of 11-*cis* retinaldehyde for regeneration. In addition, the stores of 11-*cis* retinyl ester known to exist in cattle and frog pigment epithelium [16,17] are not preferentially used for rhodopsin regeneration as compared with the esters of all-*trans* retinol [17]. It is therefore unlikely that the outer segment obtains 11-*cis* retinaldehyde as such from the pigment epithelium. A specific transport protein for 11-*cis* retinaldehyde would have to be involved, and no evidence of such a protein has been found so far.

If then 11-*cis* retinaldehyde is formed inside the outer segment, but 11-*cis* retinol cannot be oxidized by the outer segment dehydrogenase (Fig. 3) its immediate precursor, at least in cattle, must be all-*trans* retinaldehyde. This could be all-*trans* retinaldehyde released during photolysis of rhodopsin and that formed by the oxidation of all-*trans* retinol, derived from the pigment epithelium [12]. At the same time this seems to imply that the all-*trans* to 11-*cis* isomerization necessary for pigment regeneration must occur in the outer segment [6].

This leaves open the function of the 11-*cis*-specific retinol dehydrogenase of the pigment epithelium. There is another source of 11-*cis* vitamin A compounds in the pigment epithelium: rhodopsin, which is digested after apical packages of rod disks have been scavenged by the pigment epithelium. This process, described in detail by Young and Bok [18], is part of the continuous rod-renewal system, taking about 10 days in mammals and 28 days in frogs. This means that every day 1/10 of 1/28 of the rhodopsin content of the eye is taken up by the pigment epithelium and digested. Indeed, Shichi [19] has recently provided direct spectrophotometric evidence that rhodopsin and what is probably a product of its partial degradation occur in cattle pigment epithelium. It is possible that the 11-*cis* retinol dehydrogenase we have found in the pigment epithelium is involved in this process and reduces the chromophoric group, apparently still in the 11-*cis* configuration, to retinol. This in turn would be esterified to retinyl esters by an esterase, which in cattle has been shown to exhibit no geometric preference toward all-*trans* and 11-*cis* retinol [16]. This hypothesis is supported by the observations of Hubbard and Dowling [17] that in the frog the percentage of 11-*cis* isomer in the pigment epithelium pool of retinyl esters slowly increases over a period of 24 h or more in continuous darkness. The geometric stability of 11-*cis* retinyl esters, as opposed to 11-*cis* retinaldehyde and 11-*cis* retinol [6], might be related to the completely hydrophobic conditions in the lipid droplets, which are believed to contain the

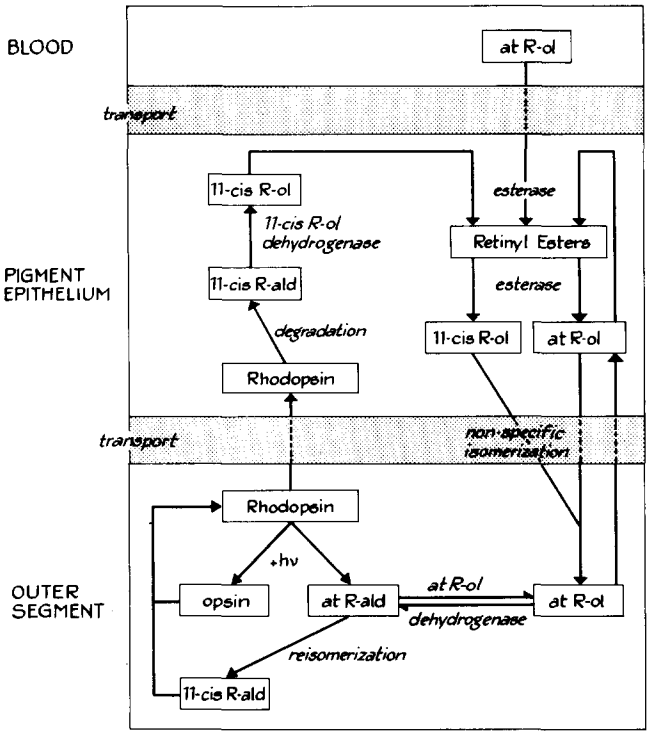

Fig. 5. Schematic representation of pathways of vitamin A compounds in the bovine eye. Abbreviations: at R-ol: all-*trans* retinol; at R-ald: all-*trans* retinaldehyde.

retinyl esters in the pigment epithelium. This store of retinyl esters is also fed by all-*trans* retinol derived from the photolysis of rhodopsin in the rod outer segments and from the blood supply. For de novo synthesis as well as for regeneration of rhodopsin the retinyl esters are hydrolyzed and both all-*trans* and 11-*cis* retinol move towards and through the outer segments. The 11-*cis* configuration is probably lost during this migration, through nonspecific isomerization [6]. Subsequently, the all-*trans* retinol is oxidized by the outer segment dehydrogenase to all-*trans* retinaldehyde, which is reisomerized to the 11-*cis* configuration and the resulting 11-*cis* retinaldehyde recombines with opsin. The latter step drives the entire series of reactions by trapping the final product, 11-*cis* retinaldehyde [1,4].

According to this formulation, summarized in Fig. 5, the function of all the enzymes that act on retinaldehyde in the eye would be to permit recycling of retinaldehyde for visual pigment formation, thereby efficiently conserving the ocular stock of vitamin A compounds essential for visual function.

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