National Institutes of Health, Research Grant No. RR01077 from the Department of Research Resources. We are also indebted to Dr. Kinuko Iwasa, Kobe Women's College of Pharmacy, for helpful discussions concerning the structure of (+)-isocorynoline.

Registry No. (±)-**1**, 68035-45-0; (±)-**2**, 82950-95-6; (±)-**3**, 83607-67-4; **10**, 66303-84-2; (±)-**11**, 82929-73-5; (±)-**13**, 83607-71-0; (±)-**14**, 82929-74-6; **15**, 63254-33-1; (±)-**16**, 82929-75-7; (±)-**17**, 83607-68-5; (±)-**18**, 85083-21-2; (±)-**19**, 85083-22-3; (±)-**20**, 82929-76-8; (±)-**21**, 82929-77-9; **22**, 85083-23-4; (±)-**29**, 83607-69-6; (±)-**30**, 83607-63-0; (±)-**31**, 83607-66-3; (±)-**32**, 85083-24-5; (±)-**33**, 85083-25-6; (±)-**34**, 85083-26-7; (±)-**35**, 83607-70-9; (±)-**36**, 83607-64-1; (±)-**37**, 83632-43-3; (±)-**38**, 83632-42-2; (±)-**39**, 83607-65-2; (±)-**40**, 85096-81-7; (±)-**41**, 85083-27-8.

Studies on the Catalyzed Interconversions of Vitamin A Derivatives

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Abstract: The kinetics of the I_2-catalyzed isomerization of the retinal isomers were studied. The all-trans isomer formed 13-*cis*-retinal rapidly with a rate constant of 1.9×10^{-4} s^{-1}. The reverse reaction occurred with a rate constant of 4.5×10^{-4} s^{-1}. The 11-cis isomer was first converted to *all-trans*-retinal with a rate constant of 3.1×10^{-4} s^{-1}, although the 13-cis isomer was also rapidly formed. The 9-cis isomer was isomerized to the 9-cis,13-cis isomer before the other isomers were generated and the 13-cis isomer was converted to its all-trans congener prior to the formation of the other isomers. Similar results appear to occur when other methods of catalysis are used. This isomerization about the C_{13}–C_{14} double bond appears to be a kinetically favored event, eliminating the possibility that 11-cis might be a kinetic product formed from the all-trans isomer. At equilibrium, only 0.1% of 11-*cis*-retinal is found. Equilibration of *all-trans*-retinol palmitate also generated very little of the 11-cis isomer (≤0.2%) 11-*cis*-retinol palmitate at equilibrium. The implications of these results for an 11-*cis*-retinal regeneration mechanism in the eye are discussed.

The stereoisomers of vitamin A aldehyde figure importantly both in visual transduction in animals and in energy production in certain bacteria. In animals, the photoisomerization of 11-*cis*-retinal to *all-trans*-retinal, both bound as protonated Schiff's bases to opsin, is the initial step in visual transduction.[1] In separate steps, the thermal resynthesis of 11-*cis*-retinal must occur for scotopic vision to proceed. In *Halobacterium halobium* the photoisomerization of the protonated Schiff's bases of *all-trans*- and 13-*cis*-retinal, bound to bacteriorhodopsin, leads to the translocation of protons across the bacterial membrane.[2]

Since the photochemical and thermal interconversions of certain stereoisomers of vitamin A aldehyde are of such great importance in biological processes, it is of some importance to gain a clear picture of the equilibrium positions of the various isomers and their rates of interconversion. As far as we are aware there are no data in the literature concerning the kinetics of retinoid interconversions and the data on the equilibrium positions seemed to depend on the method used to establish the equilibrium.[3-5] In this paper we report a study on the iodine-catalyzed equilibration and the rates of interconversion of the vitamin A aldehyde stereoisomers. In addition, we determined the equilibrium position of the retinol palmitates, the major storage form of vitamin A in the eye.[6,7]

Materials and Methods

11-*cis*-, 9-*cis*-, and 13-*cis*-retinal were generous gifts of Dr. William E. Scott of Hoffmann-La Roche Inc. *all-trans*-Retinal and *all-trans*-retinol palmitate were products of Sigma Laboratories, Inc. [15-^{3}H]-*all-trans*-Retinal of specific activity 70 μCi/μmol was synthesized by oxidizing 15-^{3}H]retinol (New England Nuclear) with freshly prepared MnO_2 by the published procedure.[8] The retinol palmitates were prepared from the desired retinol isomer and palmitoyl chloride by the published procedure.[9] The trifluoroacetic acid was a product of Aldrich Chemical Co. Chloroform and iodine were products of the Fisher Chemical Co. The chloroform was distilled before use. HPLC-grade *n*-heptane, *n*-hexane, and diethyl ether were products of Burdick and Jackson Laboratories.

High-pressure liquid chromatographic (HPLC) separations of the retinal isomers were accomplished by using a 5 μm particle size 25 cm size Hibar-II, LiChrosorb silica column (Merck, Inc.) with 10–12% ether/hexane as an eluent at a flow rate of 0.7–0.8 mL/min. A Waters Model M-6000A HPLC pump was used with either a Model 440 UV detector, containing a 365-nm filter, or a Model 480 variable-wavelength detector. The retinol palmitates were separated on the same system by using 0.5% ether/hexane as an eluent with a flow rate of 0.7 mL/min. The amounts of isomers formed in quantities >1% were measured from the areas of the peaks after correcting for the relative extinction coefficients.[10,11]

The equilibration studies were carried out as follows: The position of equilibrium was determined by using the 9-*cis*-, 11-*cis*-, 13-*cis*-, and *all-trans*-retinal isomers or the corresponding retinol palmitates in *n*-heptane with I_2 as a catalyst (0.79 μmol/mL I_2, 0.24 μmol/mL retinal or retinol palmitate at 50 °C). All manipulations were done under a red safety light, and the I_2 solutions were treated in aluminum foil wrapped extraction tubes capped under N_2. At various times a small aliquot, approximately 0.5 mL, was washed with an equal volume of a saturated sodium thiosulfite solution. The heptane layer was removed and dried over anhydrous sodium sulfate, and a standard aliquot was injected into the HPLC. The same equilibrium was reached from all four isomers.

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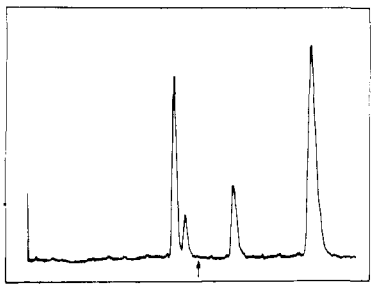


Figure 1. HPLC trace of the I_2-catalyzed equilibrium mixture of the retinals. The separation technique is described under Materials and Methods. The order of elution is the 13-cis, 9-cis,13-cis, 9-cis, and all-trans isomers. An arrow points to where the 11-cis would appear.

Table I. Equilibrium Positions of the Retinals and Retinol Palmitates[a]

		% at equilibrium				
retinoid	catalyzed	13-cis	9-cis, 13-cis	9-cis	11-cis	all-trans
all-trans-retinal	I_2	23.19	4.01	10.91	0.1[b]	61.79
all-trans-retinal	TFA	21.85	5.34	13.94	n.d. (≤0.2)	58.85
all-trans-retinol palmitate	I_2	24.05	5.17	10.20	n.d. (≤0.2)	60.55

[a] The retinoids (0.24 mM) in *n*-heptane were treated with I_2 (0.39–0.78 mM) and heated at 50 °C until equilibrium was reached. The same equilibrium position was established from the all-trans, 11-cis, 9-cis, and 13-cis isomers. n.d. = not determined (≤0.2%) under the conditions of the experiment. [b] 4×10^5 cpm of [15-^{3}H]-*all-trans*-retinal was used to determine the amount of the 11-cis isomer here.

To determine the precise position of equilibrium with the retinals, [15-^{3}H]-*all-trans*-retinal was equilibrated as above. The isomers were separated by HPLC, collected, and counted in Econofluor (New England Nuclear, Inc.) scintillant on a Beckman LS-330 liquid scintillation counter.

To establish the equilibrium by using the trifluoroacetic acid as a catalyst, 6 mg (0.021 mmol) of *all-trans*-retinal was dissolved in 0.1 mL of chloroform along with 8 mg (0.07 mmol) of the catalyst.[4] The mixture was kept in the dark for 1 h at 20 °C and worked up by diluting an aliquot with chloroform, washing with saturated sodium bicarbonate, drying over anhydrous sodium sulfate, and injecting an aliquot into the HPLC.

Kinetic studies with I_2 were carried out by using 24 μM of the retinal isomer with 5 μM I_2 in heptane at 40 °C in the dark. At the appropriate times aliquots were removed in a dim red light and the sample was prepared for HPLC analysis as indicated in the equilibrium studies.

Results

I_2-Catalyzed Equilibration of the Vitamin A Aldehydes and Palmitate Esters. The relevant isomers of vitamin A aldehyde in these studies are the 11-cis, 13-cis, 9-cis, 9-cis,13-cis, and the all-trans isomers. The isomers can be separated to base line by HPLC techniques on a silica column (see, for example, ref 4). In initial studies, it was shown that quantitatively identical HPLC patterns of the various isomers could be obtained by heating a heptane solution of either 9-cis, 11-cis, 13-cis, or all-trans isomer in the presence of I_2 in the dark. In Figure 1 a representative HPLC trace is shown. Hence, as required, the same equilibrium distribution of the aldehydes was obtained by starting from all the isomers. A virtually identical HPLC trace was also obtained by using trifluoroacetic acid as an equilibration catalyst. This is in accord with the trifluoroacetic acid catalyzed data reported by others.[4]

The amount of 11-cis could not be quantified in the I_2-catalyzed experiment since the peak was too small to reliably detect and quantitate. To quantify the actual amount of 11-cis at equilibrium, the equilibration of [15-^{3}H]-*all-trans*-retinal by I_2 in heptane was carried out. After equilibration, the mixture was applied to the HPLC column and the relevant peaks were collected and their amounts determined by liquid scintillation counting. The results are shown in Table I. What is noteworthy here is the exceedingly small amount of the 11-cis isomer at equilibrium. At 50 °C there is only 0.1% 11-*cis*-retinal at equilibrium, with a $\Delta G°_{cis/trans}$ = 4.131 kcal/mol.

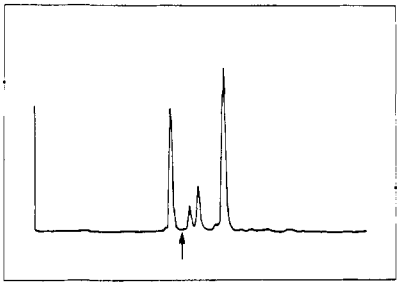


Figure 2. HPLC trace of the I_2-catalyzed mixture of the retinol palmitates. The separation technique is described under Materials and Methods. The order of elution is the 13-cis, 9-cis,13-cis, 9-cis, and all-trans isomers. An arrow points to where the 11-cis would appear.

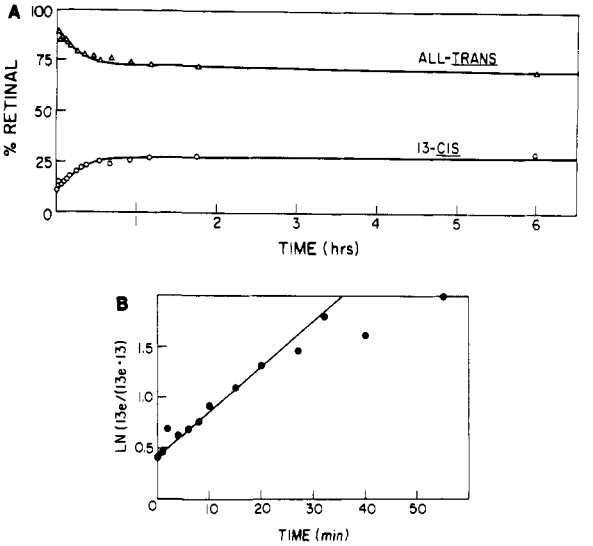


Figure 3. Iodine-catalyzed isomerization of *all-trans*-retinal. The incubations were carried out at 40 °C in the dark by using 5 μM I_2 and 24 μM *all-trans*-retinal in *n*-heptane. The percentage of the *all-trans*- and 13-*cis*-retinal isomers were determined as indicated under Materials and Methods. In (B), 13 refers to the amount of the 13-cis isomer and 13e refers to the amount of the 13-cis isomer at equilibrium.

Since retinol palmitate is the major storage form of vitamin A in the eye, it was of interest to determine the equilibrium distribution of this isomeric set. To these ends *all-trans*-retinol palmitate and 11-*cis*-retinol palmitate were equilibrated in the dark by using I_2. Figure 2 shows a typical HPLC trace of the equilibrated solution. The equilibrium values are given in Table I. Again the 11-cis isomer only accounts for a very small amount of the total (≤0.2%).

Kinetics of Vitamin A Aldehyde Isomer Interconversions. In these experiments, quantitative measurements were made on the rates of the I_2-catalyzed interconversion of the 11-*cis*- and *all-trans*-retinal isomers. These isomers and these isomers alone are found in the vertebrate visual system.[1] We have also included some qualitative data on the 9-cis and 13-cis isomers for the sake of completeness.

(a) I_2-Catalyzed Isomerization of *all-trans*-Retinal. The kinetics of isomerization of *all-trans*-retinal were relatively simple at low concentrations of I_2. Under these conditions we have found that isomerization only occurs about the 13–14 double bond. In Figure 3A data are shown on the I_2-catalyzed isomerization of the all-

$$\text{all-trans} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{13-cis}$$

trans isomer. As can be seen here, rapid equilibration occurs between the 13-cis and the all-trans isomers within approximately 30 min. This can be replotted (Figure 3B) to obtain the kinetic constants k_1 and k_2 as 1.9×10^{-4} s^{-1} and 4.5×10^{-4} s^{-1}, respec-

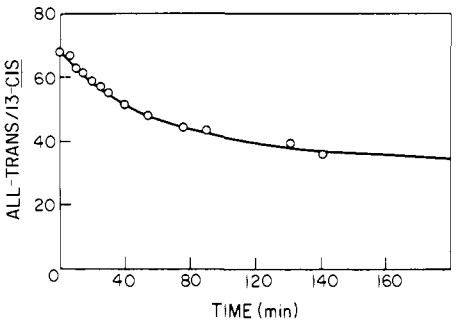


Figure 4. Iodine-catalyzed isomerization of 11-*cis*-retinal. 11-*cis*-Retinal (24 μM) was heated at 40 °C in the dark by using 5 μM I_2 as catalyzed in *n*-heptane. The workup was identical with that indicated in Figure 3.

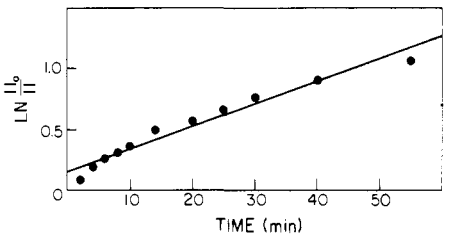
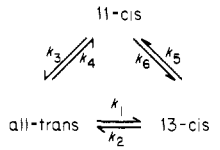


Figure 5. Iodine-catalyzed isomerization of 11-*cis*-retinal. As in Figure 4 the I_2-catalyzed isomerization of 11-*cis*-retinal was followed. In this instance the all-trans to 13-cis ratios were followed as a function of time. In the figure 11_0 refers to the amount of 11-*cis*-retinal at zero time.

tively. These data show that 11-cis formation is not kinetically favored when starting from all-trans.

(b) I_2-Catalyzed Isomerization of 11-*cis*-Retinal. The kinetics of this reaction are more complicated than those from all-trans, because three chemical species exist at any point in time, the 11-cis, 13-cis, and all-trans isomers. The general kinetic scheme is depicted as



At first glance it would appear difficult to uniquely determine the kinetic constants in this scheme. Fortunately, a few simplifying assumptions can be made here. First, the rates of formation of 11-cis from all-trans (k_4) and 13-cis (k_5) can be ignored, because no 11-cis was ever observed in the I_2-catalyzed isomerization of all-trans or 13-cis under the conditions of these experiments. Furthermore, because of the equilibrium distribution of 11-cis it would be predicted that k_4 and k_5 are negligible, relative to k_3 and k_6. It can probably also be assumed that there is no direct isomerization both between 11-cis and 13-cis. To test some of these assumptions, experiments were performed following the I_2-catalyzed isomerization of 11-*cis*-retinal. In Figure 4 the ratio of all-trans/13-cis is plotted as a function of time. The steady decline in this ratio indicated that 11-cis is isomerized to all-trans faster than it is isomerized to 13-cis, an observation consistent with the assumptions made above.

Given the assumptions made above, the kinetic scheme simplifies to

$$\text{11-cis} \xrightarrow{k_3} \text{all-trans} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{13-cis}$$

If this is true, the disappearance of 11-cis should follow a simple exponential decay curve with a time constant k_3. The straight line was determined by linear regression analysis. The data are shown in Figure 5, and k_3 was calculated to be 3.1×10^{-4} s^{-1}.

(c) I_2-Catalyzed Isomerization of 9-Cis and 13-Cis. Full-time courses were not measured here but an early time point was taken to ascertain whether there was a kinetically favored intermediate.

Table II. Isomerization of 9-*cis*- and 13-*cis*-Retinal by I_2[a]

starting material	isomer formed, %				
	all-trans	13-cis	9-cis	9,13-dicis	11-cis
9-cis	16.79	7.74	53.62	21.75	n.d.
13-cis	67.30	32.72	n.d.	n.d.	n.d.
11-cis	65.82	31.57	2.64	n.d.	n.d.
all-trans	66.90	32.56	0.37	n.d.	n.d.

[a] In the above experiments, the appropriate retinal (24 μM), in heptane plus 5 μM I_2, was heated at 40 °C for 2 h. The I_2 was destroyed by shaking with saturated sodium thiosulfate. After the mixture was dried over anhydrous sodium sulfate, the relative amounts of the various isomers were determined by HPLC [n.d. means not determined ($\leq$0.2%) under these conditions].

Table III. Trifluoroacetic Acid Catalyzed Isomerization of *all-trans*-Retinal[a]

isomer	% isomer formed at time			
	0	1 min	15 min	1 h
all-trans	>99	77.69	73.2	58.85
13-cis		21.25	19.56	21.85
9-cis		1.14	7.32	13.94
9-cis,13-cis		n.d.	n.d.	5.34
11-cis		n.d.	n.d.	$\leq$0.1

[a] In these experiments a chloroform solution of *all-trans*-retinal (0.2 M) and trifluoroacetic acid (0.7 M) was incubated in the dark at 20 °C. At the indicated times the samples were worked up as stated under Material and Methods, and the relative isomer contents were determined by HPLC. n.d. stands for not determined ($\leq$0.2%) under these conditions. It should be noted that there is significant decomposition of the retinals under the conditions of these experiments.

For comparison's sake, the same point was taken for the 11-cis and all-trans isomers.

Two interesting points emerge from Table II. First, it appears that the 9-cis,13-cis isomer is the kinetically favored intermediate in the isomerization of the 9-cis isomer. Only at a higher iodine concentration and a longer incubation time did equilibration occur. Second, 13-cis was directly isomerized to all-trans in the absence of the formation of the other isomers of retinal. This is to be expected in light of the results obtained with *all-trans*-retinal.

(d) Trifluoroacetic Acid Catalyzed Isomerization of *all-trans*-Retinal. To determine if there was anything unique about the kinetics obtained for I_2, preliminary studies were done on the rates of the trifluoroacetic acid catalyzed isomerization of *all-trans*-retinal (Table III). Again, we see here that the all-trans $\rightleftharpoons$ 13-cis equilibration occurs first, followed by the formation of the 9-cis and 9-cis,13-cis isomers. These results are similar to those obtained with the I_2-catalyzed process.

Discussion

The absorption of a photon by rhodopsin leads to the isomerization of 11-*cis*-retinal to *all-trans*-retinal, both initially bound to opsin as Schiff's bases.[1] As a result of conformational changes in the protein backbone of opsin, the *all-trans*-retinal Shiff's base is hydrolyzed, resulting in the bleaching of the pigment.[1] In order to maintain vision, reisomerization about the 11–12 double bond of *all-trans*-retinal, or a derivative thereof, must occur either in the retina or in the pigment epithelium.[7] It is likely that this isomerization is a dark or thermal process.[7] In a light-adapted animal (during strong bleaching) the percentage of the retinoids in the 11-cis form may be only 5–10% of the total.[10] However, during dark adaptation the value could climb to 50–60%.[7,10] How this process occurs remains a mystery. It is not even known if the process is enzyme mediated or not. This being the case, it is of some importance to precisely determine the equilibrium positions of the retinoids and to determine if 11-*cis*-retinal can be formed as a kinetically favored product from *all-trans*-retinal which could then be trapped as it is generated. These measurements are the subject of this article.

Two articles in the literature suggested that 11-*cis*-retinal is only a minor isomer at equilibrium.[3,4] The procedure used here

to establish equilibrium involved the treatment of *all-trans*-retinal with trifluoroacetic acid in chloroform. The first observation was a NMR study and noted that 11-*cis*-retinal was less than 5% at equilibrium.[3] The second observation involved HPLC separation of the isomerized materials and estimated that 11-*cis*-retinal was about 0.048% at equilibrium.[4] A possible problem here is that trifluoroacetic acid causes extensive degradation of the retinoids. In contrast to these experiments, the I_2-catalyzed isomerization of *all-trans*-retinal was stated to afford approximately 12.5% 11-cis at equilibrium.[5] This latter value is often quoted, as is the calculated free energy difference of 1.2 kcal/mol between the 11-cis and all-trans.[11,12] Our results here are confirmative of the trifluoroacetic acid data and extend it because the equilibrium was established by a different mechanism. It should be noted that the same equilibrium is probably reached under base catalyzed conditions. The reduced flavin catalyzed isomerization of all-*trans*-retinal probably affords the same equilibrium mixture.[13] We also determined that there was $\leq 0.2\%$ of 11-*cis*-retinol palmitate in equilibrium with its 9-*cis*-, 9-cis,13-cis, 13-cis and all-trans congeners. The palmitate esters are the predominant forms of vitamin A in the eye in addition to the retinals.[7]

The fact that the retinoids are predominantly in the 11-cis form in the dark adapted eye means that a nonequilibrium situation exists. Where the energy comes for this is not known. Although ATP could be involved, specific binding proteins which stabilize the 11-*cis* isomers are a more likely possibility.[14] This latter possibility is especially attractive since the energy difference between the 11-cis and all-trans isomers, being 4.13 kcal/mol, could easily be supplied by binding interactions.

Although the equilibrium levels of 11-*cis*-retinal are far below those found in the dark-adapted eye, it was of some interest to determine whether 11-*cis*-retinal could be a kinetically favored product. If the 11-*cis*-retinal generated chemically by a kinetically favored pathway were specifically trapped by a binding protein in the retina or pigment epithelium, the preponderance of the 11-cis isomer might be understood. This is of special interest in light of the question as to whether the interconversion of 11-*cis*-retinal and *all-trans*-retinal is an enzymatic or nonenzymatic process. The fact that only these two isomers are found in vivo suggests but does not prove that an "isomerase" exists. Furthermore, a burst of the 11-cis isomer might also explain the earlier I_2-catalyzed "equilibrium" result.[5] To these ends, we have studied the kinetics of the I_2-catalyzed isomerization of 11-*cis*- and *all-trans*-retinal. The isomerization pattern from *all-trans*-retinal was simple indeed. At low I_2 concentration a facile isomerization occurred about the C_{13}–C_{14} bond, resulting in the formation of 13-*cis*-retinal. The forward rate constant is 1.9×10^{-4} s^{-1} and the back rate constant is 4.5×10^{-4} s^{-1}. Only at higher concentrations did the other isomers of retinal form. These experiments show that 13-cis is the kinetically favored product and not 11-cis. Therefore, a chemical mechanism for 11-*cis*-retinal formation in vivo that required a kinetically controlled production of this isomer from all-trans can probably be ruled out. It is noteworthy that the facile isomerization about the C_{13}–C_{14} bond is not idiosyncratic to I_2 catalysis but is also found during acid (trifluoroacetic acid) catalysis.

Studies on the kinetic I_2-catalyzed isomerization of 11-*cis*-retinal were more complicated than the kinetics of *all-trans*-retinal because of the competing isomerization reactions. Nevertheless, it appeared that all-*trans* was the kinetically favored product which, once formed, could yield the 13-cis isomer. Interestingly, the linear limiting velocity for the 11-cis to all-trans conversion (3.1×10^{-4} s^{-1}) was actually slower than the 13-cis to all-trans conversion (4.5×10^{-4} s^{-1}). Thus, the relative stabilities of the 13-cis and 11-cis isomer are not being directly translated into the stabilities of the transition states for the conversions. Considering that the catalyzed isomerization mechanism could involve the addition of I· to retinal, this is not surprising.[15,16] If the kinetically favored mode of I· addition was to the C_{13}, with the radical being delocalized over the carbonyl group, then isomerization reactions could not occur over the remainder of the carbon skeleton of retinal. In addition, the rate-limiting reaction could occur at any number of steps, other than rotation about the isomerizing bond, such as the cleavage of the C–I bond, which restores the fully conjugated product.[15]

Although a full study of the isomerization reactions of the 13-cis and 9-cis isomer was not undertaken, some of the results are nevertheless noteworthy. First of all, the 13-cis isomer behaved as expected, generating the all-trans isomer before any of the other isomers were formed. The 9-cis isomer was transformed into the 9-cis,13-cis isomer first, before equilibration occurred. This experiment attests to the kinetic facility with which isomerization occurs about the C_{13}–C_{14} bond of retinal. It has been reported that the retinals are isomerized as a consequence of Schiff's base formation with phosphatidylethanolamine.[17] Specificity was invoked in these isomerizations partly because the 9-cis isomer was found only to "equilibriate" with its 9,13-cis congener.[17] This result is readily understandable in terms of kinetic control, where we see again the same pattern develops irrespective of the mode of catalysis.

In conclusion, our experiments show that isomerization about the C_{13}–C_{14} bond is a kinetically favored isomerization pathway in the catalyzed interconversions of the retinal isomers. 11-*cis*-Retinal is never formed preferentially by a chemical mechanism, precluding the possibility that it is generated by kinetic mechanism in the eye. Furthermore, evidence is presented which shows that only 0.1% of 11-*cis*-retinal is present at equilibrium, a result consistent with observations reported earlier using trifluoroacetic acid or a catalyst.[3,4] The equilibrium positions reached with retinol palmitate were observed to be quite similar to those observed with the retinals. These facts are, on the surface, difficult to reconcile with the known preponderance of the 11-cis isomer(s) in the eye unless this (these) isomer(s) is (are) stabilized by binding proteins or an energy source, such as ATP, is consumed during its production.

Acknowledgment. We thank the National Institutes of Health (EY-04096) for support of this research.

Registry No. *all-trans*-Retinol palmitate, 79-81-2; 9-*cis*-retinal, 514-85-2; 13-*cis*-retinal, 472-86-6; 11-*cis*-retinal, 564-87-4; *all-trans*-retinal, 116-31-4; 9,13-*cis*,*cis*-retinal, 23790-80-9; 11-*cis*-retinol palmitate, 51249-33-3; I_2, 7553-56-2.

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