Nonstereospecific Biosynthesis of 11-*cis*-Retinal in the Eye†

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ABSTRACT: [^{3}H]-*all-trans*-Retinol injected intraocularly into rats is processed to [^{3}H]-11-*cis*-retinal, the visually active retinoid that binds to opsin. After 18 h, virtually all (93%) of the radioactive retinals recovered were in the form of 11-*cis*-retinal. At earlier times, however, both *all-trans*- and 13-*cis*-retinals, the latter being a nonphysiological isomer, were formed. Both of these isomers disappeared concomitant with the formation of 11-*cis*-retinal. The rise and fall of 13-*cis*-retinal suggest that this isomer can be converted into 11-*cis*-retinal either directly or indirectly in vivo and, hence, that the biosynthesis of the latter is nonstereospecific. This hypothesis was verified by showing that in double-labeling experiments [^{14}C]-13-*cis*-retinol was converted into 11-*cis*-retinal nearly as well (~70%) as [^{3}H]-*all-trans*-retinol. These studies show that the biosynthesis of 11-*cis*-retinal can be nonstereospecific and, hence, that the process may be chemically rather than enzymatically mediated in vivo. In contrast, double-labeling studies with [^{14}C]-9-*cis*-retinol and [^{3}H]-*all-trans*-retinol showed that very little, if any, of the 9-cis isomer was processed to 11-*cis*-retinal in vivo although it did form isorhodopsin. This is consistent with what is known about the relative chemical stabilities of 9-*cis*-retinoids from model studies. The isomerization of 9-*cis*-retinoids is much slower than that of their all-trans, 13-cis, or 11-cis congeners. These results are discussed in terms of a possible mechanism for the biosynthesis of 11-*cis*-retinal in vivo and suggest that the isomerization event need not necessarily be enzyme mediated.

The absorption of a photon of light by rhodopsin with the subsequent isomerization of the 11-*cis*-retinal Schiff base to its all-trans congener is the initial event in vision (Wald, 1968; Hubbell & Bownds, 1979). The isomerization itself occurs within picoseconds and is followed by a series of slower protein conformational changes, which result in the bleaching process and the eventual hydrolysis of the Schiff base linkage to afford *all-trans*-retinal and opsin (Dratz, 1977; Uhl & Abrahamson, 1981). One of the rhodopsin conformers, probably metarhodopsin II, on the way to hydrolysis, drives the next step(s) in the visual transduction mechanism that results in the hyperpolarization of the rod outer segments (Uhl & Abrahamson, 1981).

The liberated *all-trans*-retinal is then reduced by an alcohol dehydrogenase and coupled to long-chain fatty acids (Uhl & Abrahamson, 1981). In strong light much of the rhodopsin is bleached, and of the total vitamin A, most gets stored in the pigmented epithelium as long-chain esters (Dowling, 1960). This process is referred to as photochemical adaptation (Dowling, 1967). In order for the eye to adapt to low light intensities, the functionally inactive opsin formed at the high light intensities must recombine with 11-*cis*-retinal to form rhodopsin. In the dark, the ester fraction is mobilized via the action of an esterase(s) to yield vitamin A, which is then oxidized to form vitamin A aldehyde (retinaldehyde). Somewhere in this visual cycle, reisomerization of the trans 11–12 double bond to the cis 11–12 double bond must occur. It is often assumed that this isomerization occurs at the aldehyde stage and that a specific "retinal isomerase" enzyme exists to carry out this conversion (Davson, 1980; Uhl & Abrahamson, 1981). However, in over 30 years this putative enzyme has not been isolated, nor has its activity ever been detected. None of the published reports of the existence and activity of the enzyme have withstood the test of time (Hubbard, 1956; Amer & Akhtar, 1973). A question that emerges is whether there is an enzyme in vivo that catalyzes the interconversion of the *all-trans*- and 11-*cis*-retinoids or whether, in fact, the process is simply chemical. For example, reduced flavins and phosphatidylethanolamine can be catalytic for isomerization (Futterman & Rollins, 1973; Groenendijk et al., 1980b). In fact, with secondary amines, such as piperidine, it appears that the Schiff base formation rather than isomerization is rate limiting, and in dilute solutions the thermal isomerization of the charged Schiff base can be over in a matter of minutes (Lukton & Rando, 1984). It is noteworthy that the rates of retinoid isomerization in the vertebrate eye in vivo are rather slow, dark adaptation taking 1–2 h in frogs and albino rats (Bridges, 1976; Dowling, 1960). In order to try to distinguish between enzymatic and nonenzymatic mechanisms, it is pertinent to ask whether or not the isomerization process is stereospecific in vivo. Since none of the vitamin A isomers contain asymmetric centers, this test must rely on the use of diastereomers rather than enantiomers. It is shown here that the isomerization process is nonstereospecific by showing that both *all-trans*-retinol and 13-*cis*-retinol injected intraocularly are converted into 11-*cis*-retinal in the retina of the albino rat in vivo. Furthermore, pure *all-trans*-retinol is converted into a quasi-equilibrium mixture of *all-trans*- and 13-*cis*-retinals prior to its conversion to 11-*cis*-retinal in the rat retina. These results bring into question the assumption that an enzyme mediates the isomerization event.

Materials and Methods

Unless otherwise mentioned, all procedures were performed under dim red lights with samples kept on ice.

Preparation of Radioactive Retinoids. [11,12-^{3}H]-*all-trans*-Retinol (60 Ci/mmol, Amersham) in 100-μCi quantities was repurified on a Waters HPLC[1] system using a Hibar EC,

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LiChrosorb Si 60, 5-μm (250 × 4.0 mm) silica column (Merck, Inc.) with 20% diethyl ether in hexane (Burdick and Jackson Laboratories, Inc.) as an eluant at a flow rate of 2 mL/min (Groenendijk et al., 1980c). A 313-nm filter was used for absorbance detection. The *all-trans*-retinol peak was collected with a Gilson 201 fraction collector in the peak mode. Typical isomeric purity was greater than 98%, with [^{3}H]-13-*cis*-retinol the only detectable contaminant. When coinjections were done with ^{14}C-labeled retinols, specific activities were matched by adding unlabeled *all-trans*-retinol (Fluka).

[10,11-^{14}C]-13-*cis*-Retinol (27.7 mCi/mmol) and [15-^{14}C]-9-*cis*-retinol (15.5 mCi/mmol) were prepared by reduction of the corresponding [^{14}C]retinoic acids (generous gifts of Hoffmann-La Roche) or their methyl esters with $LiAlH_4$ (Aldrich) in distilled diethyl ether (Bridges & Alvarez, 1982). The reaction was quenched with 50% methanol in water, and the product was extracted into ether. Partial purification was achieved by elution through a silica gel SEP-PAK (Waters, Inc.) cartridge. [^{14}C]-13-*cis*-Retinol was further purified with the HPLC system described above. [^{14}C]-9-*cis*-Retinol (86% pure) was not further purified as a result of the very limited supply available and poor HPLC separation between 9-*cis*-retinol and *all-trans*-retinol.

Isomeric purities of the purified retinols were confirmed on small samples by formation of their palmitate esters with palmitoyl chloride (Aldrich) in methylene chloride containing triethylamine (Bridges & Alvarez, 1982). HPLC analysis was then done in 0.5% ether in hexane at 2 mL/min. Ester analysis is required because it is impossible to achieve a base-line separation of all the alcohols by HPLC; the palmitate esters, on the other hand, separate cleanly. No detectable retinals or 11-*cis*-retinoids were ever present in any of the radioactive retinols prepared. Radioactive retinols were dissolved in ethanol containing 1 mg/mL butylated hydroxytoluene (Sigma) as an anti-oxidant and stored at −70 °C. Isomeric purity checks were done periodically, and repurification was done as necessary.

In Vivo Biosynthesis of Isomeric Retinals Analyzed by Oxime Formation. Male CD albino rats (Charles River Breeding) weighing 100–200 g were light adapted at least 0.5 h under two 15-W fluorescent lights at a distance of 10 cm. Each rat was anesthetized by an intraperitoneal injection of Avertin (Payne & Chamings, 1964) and then injected intraocularly in a dark room under dim red lights with 5 μL of an ethanolic solution containing the various retinol isomers. After the rats were dark adapted from 0.5 to 18 h, they were anesthetized with CO_2 and decapitated. The eyes were enucleated and sliced open with a razor blade, and each retina was dissected cleanly away from the vitreous and choroid. Each retina was solubilized separately in a 1.5-mL centrifuge tube containing 0.1 mL of 50 mM octyl β-D-glucopyranoside (Calbiochem) (Stubbs et al., 1976), and extraction of the protein-linked retinals as retinal oximes with NH_2OH (Sigma) was carried out following the published procedure, which has been characterized as quantitative and nonisomerizing (Groenendijk et al., 1980c). When experiments using multiple rats were performed, oximes were formed as each pair of eyes was excised in order to minimize isomerization.

The retinal oximes were dissolved in diethyl ether, dried over anhydrous sodium sulfate, and eluted through a silica gel SEP-PAK (Waters, Inc.) cartridge with two 3-mL portions of ether. The eluate was dried down under nitrogen and redissolved in a hexane solution containing nonradioactive carrier oximes of all four retinal isomers formed by treating a methanol photoisomerate of *all-trans*-retinal (Bridges & Alvarez, 1982) with NH_2OH. HPLC analysis was done on the system described above with 8% diethyl ether in hexane as an eluant at a flow rate of 2 mL/min and detection at 365 nm. The radioactive oxime peaks were collected on a Frac-100 fraction collector (Pharmacia) and counted in Soluscint-O (National Diagnostics) on a Beckman LS1800 scintillation counter interfaced with an Apple-II-Plus computer for data analysis and graphic display. Background corrections were done by subtracting counts collected from HPLC runs in which only the nonradioactive carrier was injected on the column.

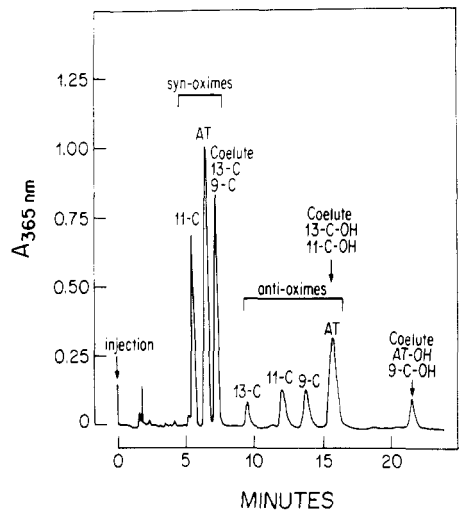

FIGURE 1: HPLC separation of retinoids. HPLC separation of retinal oximes, retinols, and retinyl esters was performed on a Waters HPLC system using a Hibar EC, LiChrosorb Si 60, 5-μm (250 × 4.0 mm) silica column. Eluant is 8% diethyl ether in hexane with a flow rate of 2 mL/min and detection at 365 nm.

Figure 1 shows a typical HPLC separation of the oximes. Only the retinal *syn*-oximes, produced in a constant ratio of at least 2:1 relative to the *anti*-oximes (Groenendijk et al., 1980a), were suitable for radioactive analysis because the unoxidized retinols have similar retention times to the *anti*-oximes. It should be noted that the 13-*cis*- and 9-*cis*-*syn*-oximes coelute. At key time points in the experiments, eyes were analyzed by an alternate extraction procedure in which retinals are extracted in their free form (Pilkiewitz et al., 1977). This method gives a much lower extraction efficiency (Groenendijk et al., 1980a) and is somewhat more prone to isomerization in our hands, but it does allow a base-line separation of all isomers of retinal. In all cases analyzed by the alternate method in which all-trans or 13-cis isomers of retinol were injected intraocularly, the 13-*cis*-retinal isomer was present in substantial excess to the 9-cis isomer; whenever 9-*cis*-retinol was injected intraocularly, 9-*cis*-retinal predominated over 13-*cis*-retinal. No significant 9-*cis*,13-*cis*-retinal was ever detected in any experiment. For the sake of clarity in this paper, counts eluting in the peak corresponding to the 13-*cis*- and 9-*cis*-*syn*-oxime peak will be referred to as 13-cis if the injected isomer was *all-trans*- or 13-*cis*-retinol and will be referred to as 9-cis if the injected isomer was 9-*cis*-retinol.

In the range of radioactivity injected (4×10^5 dpm to 3×10^6 dpm), 0.1–0.5% of the counts was recovered as retinal oximes, and no saturation effects were apparent. When less than 5×10^4 dpm of retinol was injected, the recovered radioactivity was not significantly different from background when analyzed by HPLC. Labeled retinals prepared by MnO_2 oxidation (Bridges & Alvarez, 1982) were found to have comparable uptake efficiencies into 11-*cis*-retinal pools after

[1] Abbreviations: HPLC, high-performance liquid chromatography; Con A, concanavalin A; AT, all-trans; C, cis; Rh, rhodopsin.

intraocular injection. However, the large and variable amount of unisomerized retinal recovered during isolation made data interpretation difficult, and further retinal injection experiments were not pursued. In the range of retinoid specific activities used (60 Ci/mmol to 15.5 mCi/mmol), no apparent differences in percent uptake were seen. Thus, even at low specific activity, no saturation or toxic effects were apparent. At the lowest specific activities used the labeled 11-*cis*-retinal pool, presumably bound to opsin or binding proteins, accounted for less than 5% of the eye's 1–2 nmol of rhodopsin (Dowling, 1960; Crouch et al., 1984).

Analysis of Retinols and Retinyl Esters after Intraocular Injection. The oxime extraction described above also recovers the radioactive retinols and retinyl palmitates quantitatively. Retinol peaks were usually detectable at 365 nm even without addition of nonradioactive carrier when run with 8% ether in hexane. The 13-*cis*- and 11-*cis*-retinol isomers coelute, as do the 9-*cis*- and *all-trans*-retinol isomers, and occasionally, palmitate derivatives were made to determine exact isomeric distribution. When 13-*cis*- or *all-trans*-retinol was injected into rat eyes, only 13-*cis*- and *all-trans*-retinol were present. When 9-*cis*-retinol was injected, 9-*cis*-retinol was the predominant alcohol recovered with lesser amounts of 13-cis and all-trans present.

Endogenous palmitate esters that were presumably recovered from pigmented epithelium cells isolated along with the retina were occasionally analyzed by HPLC with 0.5% ether in hexane. A base-line separation of all isomers was possible with detection at 313 nm.

In Vivo Biosynthesis of Isomeric Retinals Analyzed by Concanavalin A Binding. Six rats were light adapted, anesthetized, and injected intraocularly as previously mentioned. After these rats were dark adapted for 19 h, they were anesthetized with CO_2 and decapitated. The retinas were excised and pooled together with 0.5 mL of buffer containing 50 mM NH_2OH (pH 6.5) in a 1.5-mL centrifuge tube. After a 2-s sonication with a micro-ultrasonic cell disrupter (Kontes) on ice, the tube was centrifuged for 10 min at 13000*g* and the supernatant transferred to a scintillation vial for counting. Next, 0.5 mL of buffer including 2% Ammonyx-LO (Onyx Chemical Co.) was added to solubilize rhodopsin and isorhodopsin, and after centrifugation, the supernatant was collected. After a suitable aliquot was set aside in order to make oximes, the remaining supernatant was divided into two equal (respective to radioactivity) fractions. One fraction, A, was subjected to direct orange light (Corning 3-68 filter) at 0 °C for 15 min, and the other fraction, B, was wrapped in foil and kept on ice in the meantime. Then, to both fractions A and B, a 10-fold excess of concanavalin A–Sepharose 4B (Sigma) was added. Upon binding of visual pigment (Defoe & Bok, 1983), the concanavalin A in each fraction was extensively washed with detergent-containing buffer until a flat base line of radioactivity was reached (nine washes for each fraction). Rhodopsin, isorhodopsin, and opsin were eluted with 0.5-mL washes of 0.5 M methyl α-D-mannopyranoside (Calbiochem) in the detergent buffer. Five consecutive batchwise elutions were sufficient, and each supernatant was counted in Hydrofluor (National Diagnostics).

Results

In Vivo Processing of [^{3}H]-all-trans-Retinol in the Rat Retina. Initial experiments were performed to determine whether or not *all-trans*-retinol administered by intraocular injection could be converted into 11-*cis*-retinal in vivo in the rat. Light-adapted male albino rats were injected intraocularly in the dark with 1.7×10^6 dpm of [^{3}H]-*all-trans*-retinol (53.5 mCi/mmol) in 5 μL of ethanol, and they were allowed to dark adapt for 0.5–18 h. After decapitation, their eyes were removed, and retinas were obtained. Retinoids were extracted as described under Materials and Methods, and isomeric composition of the labeled *syn*-oxime derivatives of the retinals was analyzed by HPLC.

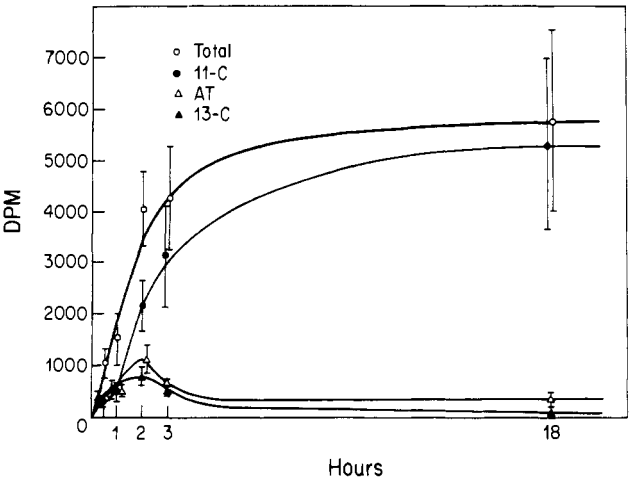

FIGURE 2: Retinal biosynthesis from [^{3}H]-*all-trans*-retinol. Light-adapted rats were injected intraocularly with 1.7×10^6 dpm of [11,12-^{3}H]-*all-trans*-retinol (53.5 mCi/mmol, 98% isomeric purity) in 5 μL of ethanol. Dark adaptation proceeded for the indicated period at which time retinals formed in vivo were isolated as their oxime derivatives. *syn*-Oxime peaks were separated by HPLC and counted for radioactivity. All values are ±SEM for five to eight eyes.

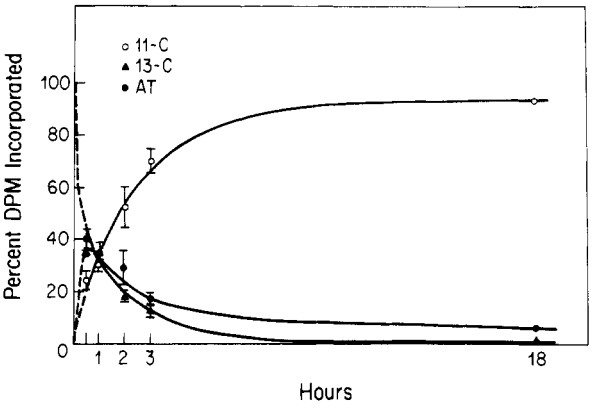

FIGURE 3: Percent retinal biosynthesis from [^{3}H]-*all-trans*-retinol. The experiment shown in Figure 2 is expressed as each isomer's percentage of total counts isolated as *syn*-retinal oximes in a particular eye. All values are ±SEM for five to eight eyes.

As can be seen in Figure 2, there is a rapid production of retinals from the injected *all-trans*-retinol that appears to level off after 5–10 h. In Figure 3 for purposes of exposition, each isomer's percentage of the retinal pool is expressed as a function of time. 11-*cis*-Retinal is the predominant isomer formed, accounting for 93% of all the aldehydes at 18 h. Presumably, all of the 11-*cis*-retinal is bound to rhodopsin or retinal binding proteins, as free 11-*cis*-retinal is highly unstable (Groenendijk et al., 1980b). The transient production of 13-*cis*-retinal is of special interest because it means that this nonphysiological isomer can be generated from *all-trans*-retinal or *all-trans*-retinol followed by oxidation. The subsequent decay of 13-*cis*-retinal concomitant with the rise in 11-*cis*-retinal suggests that the former can be converted into the latter probably via *all-trans*-retinal. The bulk of *all-trans*-retinal appears to be rapidly formed in vivo subsequent to the injection of [^{3}H]-*all-trans*-retinol. The *all-trans*-retinal thus formed could serve as a precursor for 11-*cis*-retinal. The purified

Table I: In Vivo Biosynthesis of Isomeric Retinals Analyzed by Oxime Formation

incubation time (h)	starting isomer	amount injected	amount incorporated in retinals (dpm)[a] 11-cis	all-trans	13-cis	9-cis
3	[10,11-^{14}C]-13-*cis*-retinol	1.4 × 10^6 dpm/23 nmol	1567 ± 467	457 ± 48	323 ± 49	
			63 ± 6[b]	22 ± 4[b]	15 ± 2[b]	
	[11,12-^{3}H]-*all-trans*-retinol	1.7 × 10^6 dpm/23 nmol	3224 ± 927	718 ± 89	512 ± 92	
			70 ± 5[b]	18 ± 3[b]	12 ± 2[b]	
			0.58 ± 0.03[c]	0.79 ± 0.03[c]	0.79 ± 0.07[c]	
18	[10,11-^{14}C]-13-*cis*-retinol	4.4 × 10^5 dpm/4.4 nmol	827 ± 168	239 ± 86	304 ± 145	
			66 ± 8[b]	16 ± 2[b]	18 ± 6[b]	
	[11,12-^{3}H]-*all-trans*-retinol	8.9 × 10^5 dpm/7.2 nmol	2200 ± 382	427 ± 96	373 ± 126	
			75 ± 3[b]	14 ± 1[b]	12 ± 2[b]	
			0.76 ± 0.03[c]	1.03 ± 0.18[c]	1.35 ± 0.30[c]	
19	[15-^{14}C]-9-*cis*-retinol	1.0 × 10^6 dpm/29 nmol	494 ± 48	326 ± 42		1538 ± 93
			21 ± 1[b]	14 ± 2[b]		65 ± 2[b]
	[11,12-^{3}H]-*all-trans*-retinol	9.0 × 10^5 dpm/29 nmol	2827 ± 162	354 ± 55	294 ± 41	
			81 ± 2[b]	10 ± 2[b]	9 ± 1[b]	
			0.16 ± 0.01[c]	0.84 ± 0.03[c]		

[a] All values ± SEM for three to four eyes. [b] Refers to the percent of total retinals isolated as the isomer in individual eyes. [c] Refers to the efficiency of ^{14}C vs. ^{3}H incorporation for each individual isomer (normalized to amount injected).

[^{3}H]-*all-trans*-retinol utilized in these experiments contained <2% [^{3}H]-13-*cis*-retinol by HPLC analysis. However, it would be unlikely that the 13-*cis*-retinal in the eye is produced via uptake of this <2% [^{3}H]-13-*cis*-retinol contaminating the injection mixture. In experiments described below, the efficiency of uptake of 13-*cis*-retinol into the retinal pool is even less than that of all-trans. The amount of retinals derived from [^{3}H]-13-*cis*-retinol injected (<35 000 dpm) would not be detectable above background. It should be noted that there was no detectable [^{3}H]-11-*cis*-retinol in the injected [^{3}H]-*all-trans*-retinol. However, even under equilibrating conditions only 0.1% 11-*cis*-retinol could exist (Rando & Chang, 1983).

When dark-adapted rats were injected with [^{3}H]-*all-trans*-retinol and allowed to remain in the dark for 2–18 h, qualitatively similar patterns of uptake were seen, but 50% fewer counts were taken up. The large amount of opsin available during the 1–2-h process of dark adaptation may enhance the oxidation of retinol to retinal. In all further experiments, light-adapted animals were injected to maximize uptake. When rats were light adapted, injected intraocularly in the dark, and then placed under the light for 18 h, less than 10% of the [^{3}H]-11-*cis*-retinal was recovered as compared with rats who were dark adapted after injection. Hence, light-adaptation conditions will eliminate the [^{3}H]-11-*cis*-retinal by bleaching.

Of the radioactive retinol injected into the eye, only 0.1–0.5% of the counts was recovered as radioactive retinal derivatives, and another 1–2% was present as retinyl esters. When the radioactive esters were examined in the eyes of the injected rats, they were found to coelute almost exclusively with *all-trans*-retinyl palmitate under all conditions. At time periods of 3 h or less, approximately 50% of the injected counts were recovered as the retinols associated with the retina. Importantly, the *all-trans*-retinol recovered from the retina after 3 h largely retained its stereochemical integrity (>95%). After an extended period of time (18 h), the amount of retinols isolated from the retina was substantially lower than that at 3 h. It appears that the excess free retinols that are not ordinarily present in the albino rat eye were cleared over a period of many hours. When experiments were performed to determine crossover of radioactivity from an injected eye to the contralateral uninjected eye, an insignificant number of counts was recovered in the latter. Presumably, the radioactive retinoids cleared from the eye were either excreted or stored in the liver.

In Vivo Processing of [^{14}*C*]*-13-cis-Retinol and* [^{3}*H*]*-all-trans-Retinol in the Rat Retina.* In order to determine whether or not the biosynthesis of 11-*cis*-retinol was stereospecific, the processing of 13-*cis*-retinol was studied. Double-labeling experiments using [^{14}C]-13-*cis*-retinol and [^{3}H]-*all-trans*-retinol were performed to quantitate the relative abilities of the two isomers to enter the 11-*cis*-retinal pool. Mixtures of the two isomers of equivalent amount and specific activity were coinjected into the eyes of light-adapted albino rats. Only the 11-cis and all-trans isomers of retinol have been detected in the rat eye (Zimmerman, 1974). The rats were allowed to dark adapt for 3 or 18 h. In the short-term experiments (Table I), 0.17% of the injected [^{14}C]-13-*cis*-retinol counts was oxidized to retinals while the corresponding figure was 0.26% for counts derived from [^{3}H]-*all-trans*-retinol. Thus, the relative efficiency of retinal formation from 13-*cis*-retinol vs. *all-trans*-retinol was approximately 64%. In the long-term experiments, this relative efficiency rose to 92%. The distributions of counts among the retinal isomers were qualitatively equivalent whether the source was 13-*cis*-retinol or *all-trans*-retinol in the short term of long term. The injected [^{14}C]-13-*cis*-retinol contained less than 5% [^{14}C]-*all-trans*-retinol, and isomerization of [^{14}C]-13-*cis*-retinol to [^{14}C]-*all-trans*-retinol in the retina was approximately 8% after 3 h and approximately 19% after 18 h. These data demonstrate that the biosynthesis of 11-*cis*-retinal in the retina can be nonstereospecific.

Of further interest here is the analysis of the radioactive ester pools. The [^{3}H]-*all-trans*-retinol was esterified with complete stereochemical integrity within the limits of the assay system. On the other hand, the [^{14}C]-13-*cis*-retinol esterified was largely (>80%) present as [^{14}C]-*all-trans*-retinyl palmitate with the remainder being [^{14}C]-13-*cis*-retinyl palmitate. Finally, it is noteworthy that the amounts of ^{14}C and ^{3}H recovered as retinyl esters were not appreciably different. Levels of radioactivity in [^{14}C]-13-*cis*-retinol and [^{3}H]-*all-trans*-retinol in the retina and vitreous were comparable at 3 h and also at 18 h, showing no preferential depletion of either isomer.

In Vivo Processing of [^{14}*C*]*-9-cis-Retinol and* [^{3}*H*]*-all-trans-Retinol in the Rat Retina.* To further investigate the stereospecificity of 11-*cis*-retinal biosynthesis, we studied the possible conversion of 9-*cis*-retinol, another nonphysiologic isomer in the retina. Double-labeling experiments using [^{14}C]-9-*cis*-retinol and [^{3}H]-*all-trans*-retinol were conducted as described above for the 13-cis/all-trans pair. [^{14}C]-9-*cis*-Retinol was coinjected with [^{3}H]-*all-trans*-retinol into light-adapted male albino rats. Specific activity and total amount of counts were equivalent, and dark adaptation was allowed to proceed for 19 h. Efficiency of uptake of counts from

Table II: Oxime Analysis of Retinals in the Extract of the Experiment of Figure 4

incubation time (h)	starting isomer	amount injected	amount incorporated in retinals (dpm) 11-cis	all-trans	13-cis	9-cis
19	[15-^{14}C]-9-*cis*-retinol	1.4×10^6 dpm/41 nmol	180	153		1274
	[11,12-^{3}H]-*all-trans*-retinol	1.4×10^6 dpm/41 nmol	1288	283	220	
			0.14[a]	0.54[a]		

[a] Refers to the efficiency of ^{14}C vs. ^{3}H incorporation for each individual isomer (normalized to amount injected).

[^{14}C]-9-*cis*-retinol into the total retinals was 60–100% of those from [^{3}H]-*all-trans*-retinol (Tables I and II). In contrast to the results obtained with [^{14}C]-13-*cis*-retinol, marginal ^{14}C incorporation into the 11-*cis*-retinal pool was observed while 65% of the retinal counts was present as the 9-cis isomer (Table I).

This low level of radioactivity in the [^{14}C]-11-*cis*-retinal pool (0.16 efficiency of ^{14}C vs. ^{3}H incorporation) can probably be accounted for by contamination from [^{14}C]-*all-trans*-retinol and [^{14}C]-13-*cis*-retinol, 11 and 3% respectively, in the starting material. Further purification of the [^{14}C]-9-*cis*-retinol was not possible due to its extremely limited supply as well as the fact that it is poorly separated from *all-trans*-retinol by all available HPLC methods. Thus, it appears that either a very small amount or no [^{14}C]-9-*cis*-retinol was converted to 11-*cis*-retinal.

Concanavalin A Analysis after a [^{14}C]-9-cis-Retinol and [^{3}H]-all-trans-Retinol Coinjection. In order to determine whether the [^{14}C]-9-*cis*-retinal formed in the previous experiment was bound to opsin as isorhodopsin, the [^{14}C]-9-*cis*-retinol and [^{3}H]-*all-trans*-retinol coinjection described above was repeated. This time, however, instead of extracting the retinals as the oxime derivative, the visual pigments from six eyes were solubilized intact in Ammonyx-LO. After an aliquot was removed for the standard oxime analysis, the remaining detergent extract was divided into two equal parts, one of which was bleached under orange light and one of which was not. The orange filter (<0.5% transmitance below 507 nm as specified by the manufacturer) would be expected to bleach isorhodopsin and rhodopsin efficiently since they have broad absorbance peaks with λ_{max} of 487–499 nm (Hubbard et al., 1971). The only other known glycoprotein that binds retinoids, interstitial retinol-binding protein, has a λ_{max} of 350 nm (Liou et al., 1982) and would not be expected to bleach and release any bound retinoids. Oxime analysis of the retinals showed equivalent amounts of ^{14}C and ^{3}H recovered, but only 14% as many ^{14}C counts as ^{3}H counts were present in the 11-*cis*-retinal pool (Table II). Visual pigments were then bound to concanavalin A, and unbound counts were washed off. Bound visual pigments, rhodopsin and isorhodopsin, were then eluted with methyl α-mannoside. As apparent from Figure 4, 50% of the ^{14}C counts relative to the ^{3}H counts were bound to the lectin. Furthermore, as expected, both the ^{3}H and ^{14}C counts did not bind to concanavalin A if the visual pigments were first bleached with orange light. Therefore, a significant proportion, approximately 50%, of the observed [^{14}C]-9-*cis*-retinal was probably bound as isorhodopsin. The lower recovery of ^{14}C relative to ^{3}H found bound to opsin when oxime analysis showed equivalent dpm in the total retinal pool can be accounted for in several ways: (1) some [^{14}C]-9-*cis*-retinal could have been complexed to a retinal binding protein that was not bound by concanavalin A; (2) isorhodopsin may have been less stable than rhodopsin to the experimental protocol; (3) some of the [^{14}C]-9-*cis*-retinal may not have been protein bound, as model studies have shown unbound 9-*cis*-retinal is much more stable than unbound 11-*cis*-retinal (Lukton & Rando, 1984).

Discussion

The object of the studies reported here was to determine whether or not the biosynthesis of 11-*cis*-retinal occurs stereospecifically in the rat eye. It was imperative first to establish that intraocularly injected [^{3}H]-*all-trans*-retinol could indeed by converted to 11-*cis*-retinal in the rat eye in the dark. It has previously been shown in frogs that about 2% of intraocularly injected [^{3}H]-*all-trans*-retinol is esterified as *all-trans*-retinyl palmitate within 3–15 h (Fong et al., 1983). We have confirmed that a similar amount of esterification (1–2%) occurs in the rat eye. Unlike the previous study, we also examined retinals formed in the dark from [^{3}H]-*all-trans*-retinol injected into previously light-adapted animals. Figures 2 and 3 show that retinal was produced for several hours after injection. At late time points 11-*cis*-retinal accounted for greater than 90% of all retinals. This shows clearly that biosynthesis of 11-*cis*-retinal can be achieved from exogenous *all-trans*-retinol. In model studies without opsin or retinal binding protein present the 11-*cis*-isomer never accounts for greater than 0.1% at chemical equilibrium (Rando & Chang, 1983). The fact that 11-*cis*-retinal is 4.1 kcal/mol less stable than its all-trans congener (Rando & Chang, 1983) requires an energy source, and hence, a physiological mechanism for the biosynthesis of the former is obligate. It can be argued that intraocular injection of retinoids does not sufficiently mimic the physiology of endogenous retinoids, most of which are always protein-bound in the rat retina, but it is nevertheless clear that we have been able to achieve the biosynthesis of 11-*cis*-retinal bound to opsin from exogenous retinoids.

At the early time points a peak of 13-*cis*-retinal was present in the radioactive aldehyde pool (Figures 2 and 3). It appeared that the nonphysiological 13-cis isomer was then converted either directly or indirectly into its 11-cis congener. In order to confirm that 13-*cis*-retinal could indeed be converted into 11-*cis*-retinal in vivo, coinjection of [^{14}C]-13-*cis*-retinol with [^{3}H]-*all-trans*-retinol was done. The efficiency of production of total retinals from 13-*cis*-retinol was 64–92% of the efficiency of production from *all-trans*-retinol, and isomeric compositions of the retinals formed were qualitatively the same. These results show that 13-*cis*-retinol can be converted in vivo to 11-*cis*-retinal. By contrast, when [^{14}C]-9-*cis*-retinol was coinjected with [^{3}H]-*all-trans*-retinol, very little, if any, conversion to 11-*cis*-retinal was observed. The [^{14}C]-9-*cis*-retinol was, however, oxidized to 9-*cis*-retinal, some of which was probably bound to opsin as isorhodopsin.

The results reported here show that 11-*cis*-retinal can be synthesized in vivo totally in the dark after intraocular injections of *all-trans*- and 13-*cis*-retinoids while 9-*cis*-retinoids are used very slowly if at all. These studies show that the in vivo biosynthesis of 11-*cis*-retinal can occur in a nonstereospecific fashion, and hence, the possibility that the isomerization process is chemically rather than enzymatically mediated must be entertained. If an isomerase enzyme exists, then it would, of necessity, be required to bind and process at least the all-trans, 11-cis, and 13-cis isomers of a retinoid while being much less efficient with the 9-cis isomer. An

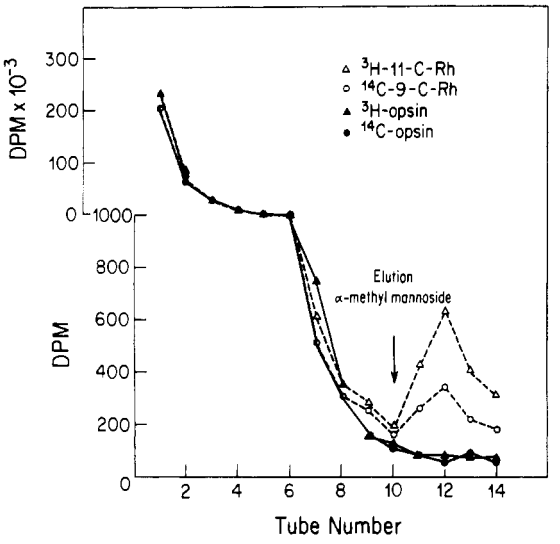

FIGURE 4: Con A binding analysis of radioactively labeled visual pigments. Con A binding analysis was performed after a coinjection of [^{3}H]-*all-trans*-retinol and [^{14}C]-9-*cis*-retinol (minimum isomeric purity 86%). The procedure was as described under Materials and Methods. Tubes 1–10 are counts that were not bound to Con A. Elution with methyl α-mannoside releases bound counts. Rhodopsin (Rh) data points and opsin data points are unbleached and bleached samples, respectively. The difference represents radioactive retinoids bound as visual pigments to opsin. Table II is an oxime analysis of a portion of the same sample used in this experiment.

enzymatic ability to process the 13-cis isomer efficiently, but not the 9-cis isomer, would be obscure since neither isomer is present in more than trace amounts in the retina.

Does the fact that 9-*cis*-retinol is weakly, if at all, converted into 11-*cis*-retinal weaken or negate the proposal that the isomerization process might be nonenzymatic in vivo? The answer is no when one considers the relative rates of isomerization of the retinals. Model studies have shown that 11-*cis*-, 13-*cis*-, and *all-trans*-retinoids are isomerized rapidly, but the 9-*cis* isomer is very resistant to isomerization with a first-order rate constant some 50 times slower than the other isomers (Lukton & Rando, 1984). It is also the last isomer to form during chemical equilibration. Thus, the transient production of 13-cis isomers and their subsequent efficient utilization as contrasted with the behavior of 9-*cis*-retinoids can be satisfactorily explained by a chemical isomerization mechanism. Furthermore, model studies show that the isomerization of the retinals is acid–base catalyzed (Lukton & Rando, 1984). Base attack at the C_{11} required for isomerization at C_{11}–C_{12} would of necessity also labilize the C_{13}–C_{14} bond and render it susceptible to isomerization. Isomerization at C_9–C_{10} would not be affected by this attack.

If the possibility of a chemically, rather than enzymatically, mediated retinoid isomerization process is to be entertained, why is the 13-cis isomer of vitamin A never observed in the eye in significant concentrations? This could be explained by the presence of specific binding proteins for the 11-cis isomer, which would remove it as it is formed. The net result of this specific stabilization in an otherwise freely equilibrating situation would be to cause the 13-*cis*-retinoids to continue to convert to 11-*cis*-retinoids and so be depleted. Certainly, some form of specific stabilization of 11-*cis*-retinal is required anyway, because it accounts for greater than 90% of all retinoids in the dark-adapted eye of the albino rat (Dowling, 1960). Without an energy source, the 11-cis isomer could not predominate whether enzymatically formed or not, because it is found to the extent of only 0.1% when the retinoids are brought into equilibrium by chemical means (Rando & Chang, 1983). Proteins that specifically bind 11-*cis*-retinal(ol) reversibly have been reported to be present in the retina (Saari & Bredberg, 1982). These binding proteins along with opsin could be at least some of the source of both the energy and the selectivity of the process that drives the *all-trans*-retinoid to 11-*cis*-retinoid conversion in vivo.

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Registry No. *all-trans*-Retinol, 68-26-8; 11-*cis*-retinal, 564-87-4; 13-*cis*-retinal, 472-86-6; *all-trans*-retinal, 116-31-4; 13-*cis*-retinol, 2052-63-3; 9-*cis*-retinol, 22737-97-9.

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