High-pressure liquid chromatography of fatty acid esters of retinol isomers
Analysis of retinyl esters stored in the eye
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We have synthesized the all-trans, 13-cis, 11-cis, and 9-cis retinyl esters of some or all of the following fatty acids: caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), arachidic (20:0), palmitoleic (16:1), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4), and docosahexaenoic (22:6). Mixtures of these compounds were analyzed by high-pressure liquid chromatography on an Altex 100 chromatograph. This approach has enabled us to investigate and identify both the isomeric form and fatty acid basis of the retinyl esters present in rabbit and rat ocular tissues. Dark-adapted rabbits had 11-cis and all-trans retinyl esters, whereas light-adapted albino rats had only the all-trans isomer. The fatty acids utilized for esterification were almost wholly palmitic for the rabbit, whereas both stearic and palmitic were present in the rat esters. Rabbits are similar to frogs in that a large proportion (80%) of their ocular retinyl esters occurred in large, fluorescent oil droplets in the pigment epithelium. These are absent in rats.

Key words: high-pressure liquid chromatography, normal phase, fatty acid esters, retinol isomers, pigment epithelium, rat, rabbit

Vitamin A occurs in the form of cis-trans isomers of the alcohol (retinol), aldehyde (retinal), acid (retinoic acid), and ester (retinyl ester).* Usually, appreciable amounts of vitamin A are contained in dark-adapted ocular tissues. In frogs 96% of this vitamin A is in the pigment epithelium (where it amounts to about 2 molar equivalents of the retinal rhodopsin). As much as 99% is esterified, and up to 55% is in the form of the 11-cis isomer. The function of these supplies is unclear. Because they are virtually absent in the albino rat eye, they do not seem to be essential for normal visual function. In the light, retinol flows from the retina into the pigment epithelium, where it is esterified and pooled with any existing stores. These retinyl esters are the precursors of 11-cis retinal used for visual pigment regeneration during dark adaptation. This process has not been elucidated. It has been suggested, however, that vitamin A returns to the retina in the esterified form and is hydrolyzed in the rod outer segment. Consequently, the

*"Vitamin A" is used here as a generic term to designate both the alcohol and its derivatives.
transport, synthesis, and hydrolysis of retinyl esters probably plays a critical role in the visual cycle, and the identification of these esters and their isomeric configuration is essential for providing an insight into how the cycle functions.

The purpose of the present study was to synthesize a series of saturated and unsaturated fatty acid esters of the four major retinol isomers, to use high-pressure liquid chromatography (HPLC) to establish conditions for separating and identifying them, and to use these data to examine the efficacy of the method for identifying the isomeric form and fatty acid basis of retinyl esters in mammalian ocular tissues. The saturated fatty acid esters synthesized ranged from C8 to C20, the series including the stearate and palmitate. The unsaturated esters synthesized included palmitoleate, oleate, and those based on the essential fatty acids, i.e., linoleate (18:2), linolenate (18:3), arachidonate (20:4), and docosahexaenoate (22:6). The four major vitamin A isomers were investigated because ocular vitamin A compounds do not occur exclusively in the 11-cis and all-trans configurations. Some 9-cis isomer is formed under certain conditions (e.g., see ref. 10), and appreciable amounts of 13-cis isomer have been observed in postmortem human pigment epithelium tissues11 and rod outer segment membrane preparations.12

HPLC has been used previously for the analysis of complex mixtures of some retinyl esters, aldehydes, and alcohols,13 11-cis and all-trans retinyl esters from frog eyes,6 and retinol isomers in amphibian and fish eyes14 as well as in synthetic mixtures of retinyl palmitate isomers.15

Although the retinyl esters synthesized by retinal tissue from exogenous all-trans retinol have been examined with regard to their fatty acid composition,16 no comparable study has been carried out on the stores found in the pigment epithelium. The data obtained from our present HPLC studies enabled us to identify both the isomeric configuration and fatty acid basis of the retinyl esters extracted from rat and rabbit pigment epithelia. Rabbit eyes were found to store retinyl esters in the dark-adapted state. On the other hand, it was necessary to light-adapt our rats because virtually no retinyl esters could be extracted from the eyes of dark-adapted animals (compare refs. 8 and 9).

Materials and methods

**Reagents.** All-trans retinol was purchased from Sigma Chemical Co., St. Louis, Mo.; 9-cis and 13-cis retinals from Eastman Kodak Co., Rochester, N.Y.; 11-cis retinal was a gift from Hoffman-La Roche, Fairlawn, N.J. The corresponding alcohols were derived from the aldehydes by NaBH₄ reduction.13,14 Fatty acid chlorides were obtained from Sigma, Aldrich Chemical Co., Inc., Milwaukee, Wisc., or NuChek Prep, Elyssian, Minn. Alumina Woelm (basic) activity grade I was from Woelm Pharma, Eschwege, West Germany. HPLC solvents were purchased from Burdick & Jackson Laboratories, Inc., Muskegon, Mich.

**Preparation of authentic retinyl esters.** The appropriate isomer of retinol dissolved in methylene chloride was incubated with the required fatty acid isomers, i.e., linoleate (18:2), linolenate (18:3), arachidonate (20:4), and docosahexaenoate (22:6). The four major vitamin A isomers were investigated because ocular vitamin A compounds do not occur exclusively in the 11-cis and all-trans configurations. Some 9-cis isomer is formed under certain conditions (e.g., see ref. 10), and appreciable amounts of 13-cis isomer have been observed in postmortem human pigment epithelium tissues11 and rod outer segment membrane preparations.12

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**Preparation of authentic retinyl esters.** The appropriate isomer of retinol dissolved in methylene chloride was incubated with the required fatty acid isomer in the presence of triethylamine or pyridine.13 After the ester had been extracted and subjected to initial purification on alumina,13 the material was stored in petroleum ether at freezer temperatures in darkness under argon. The identity of the 20:4 and 22:6 derivatives* was verified by gas chromatography after final purification by HPLC. No isomerization occurred during esterification, as judged by HPLC analysis. Subsequent saponification of the esters (for method see ref. 14) yielded retinol in its original isomeric configuration.

**Ocular tissues.** Albino rats (Sprague-Dawley) were light-adapted for 4 hr in a white tank under two 100 W incandescent lamps. The eyes were then removed, and the anterior halves (including lenses) were sliced off. The retina was dissected away from the eyecup under Binger's solution. The eyecups were ground with a small quantity of silica sand and extracted twice with acetone.8 The efficacy of this procedure was determined by a final extraction with chloroform:methanol (2:1). Virtually no vitamin A was present in this extract, as judged by its absorption spectrum, the Carr-Price reaction,14 and HPLC. After the total vitamin A present had been measured (see below), the acetone extracts were evaporated to dryness with a

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stream of nitrogen and dissolved in 10% dioxane/hexane. Highly polar constituents such as phospholipids, which cause deterioration of hplc column performance, were removed by chromatography on a 1 by 2 cm alumina column (weakened with 5% water). Vitamin A compounds were eluted with 15 ml of 10% dioxane in hexane.

Pigmented and albino rabbits were dark-adapted overnight. All operations at this stage were carried out under dim red light. After enucleation of the eyes and removal of the anterior halves, the eyecups were usually left to stand in Ringer's for about 30 min. This facilitated removal of the retina. After the retina had been dissected away, the combined pigment epithelium and choroid was scraped out and homogenized in Ringer's solution (five eyes per 4 ml) in a glass-glass tissue grinder. The homogenate was overlaid with 4 to 5 ml of petroleum ether and centrifuged at 16,000 × g for 40 min. This technique floats the pigment epithelium oil droplets to the fluid interface, where they dissolve in the petroleum ether (cf. ref. 14, where the same method was used for frog tissue). After removal of the upper phase, the operation was repeated once more. The residual pellet was then extracted with acetone. Further manipulations were identical with those for the rat extracts.

**Saponification.** This was carried out as previously described. Hplc, according to procedures described previously, showed that the isomeric configuration was retained during this operation.

**Hplc.** The instrument consisted of an Altex 100 research chromatograph with an Altex solvent programmer and Waters U6K injector. An Altex 5 μm Ultrasphere column (250 by 4.6 mm) was used. The specified minimum plate count was 15,000. The mobile phase consisted of variable amounts of diethyl ether/hexane or dioxane/hexane at flow rates specified in Results. Recovery of injected esters was typically 80% to 90% (see also ref. 13). An Altex-Hitachi 155-10 variable wavelength detector was attached to the column outlet, and the output was processed through a Columbia Scientific Supergator III. Each vitamin A compound was usually injected in an amount ranging from 0.3 to 2.0 nmol in a 10 μl volume.

**Total vitamin A.** The vitamin A present in ocular tissue extracts was measured by the Carr-Price reaction, which is independent of isomeric configuration and degree of esterification. This procedure was carried out on the extract prior to further manipulations. In the present work hplc was not used primarily to quantitate amounts of vitamin A but to identify isomeric and fatty acid composition of retinyl esters. Such quantitation can be carried out in a manner that is independent of such factors as off-column recovery, if known quantities of vitamin A compounds are injected and related to corresponding peak areas.
Results and discussion

Elution order

Fatty acid chain length and degree of unsaturation. Irrespective of the isomeric configuration of the retinyl moiety, the various fatty acid esters eluted in the same order. Retention time was related to chain length. In the series of saturated compounds the ester of arachidic acid (20:0) eluted first, followed in order by esters of stearic (18:0), palmitic (16:0), myristic (14:0), lauric (12:0), capric (10:0), and caprylic (8:0) acids.

The retention time was increased by increasing the degree of unsaturation. For the 18-carbon series the order of elution was 18:0, 18:1, 18:2, and 18:3. Similarly, for the 16-carbon series 16:1 had a longer retention time than 16:0. This is illustrated in Fig. 1, which is a representative chromatogram of the 11-cis retinyl esters of stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), palmitic (16:0), and palmitoleic (16:1) acids.

The longest retention time of all the derivatives examined in this series was obtained with the highly unsaturated 22:6 ester.

The opposing effects of increasing unsaturation and increasing chain length make it difficult to separate the 18:3 and 20:4 all-trans retinyl esters.

Effect of retinyl isomer. For any given fatty acid ester, the order of elution was 13-cis 11-cis, 9-cis, and all-trans. This is shown in Fig. 2, which illustrates near-baseline separation of these four isomers of retinyl stearate.

Resolution of different isomers and their esters: derivation of relative retention times. Although absolute retention times often varied, depending on a number of factors that included the length of time that eluant had been pumped through the column, the relative retention times were highly reproducible. We used Fig. 2 as a "standard run", where the retention times of the 11-cis, 13-cis, and 9-cis stearate esters (18:0) were ex-
Fig. 3. Summary diagram of retinyl ester retention times (in ether/hexane) expressed as percentages of the retention time of all-trans retinyl stearate (18:0). Connecting lines indicate compounds that cannot be resolved in our system, e.g., 11-cis linolenate, 9-cis linoleate, all-trans oleate, and all-trans laurate. As noted in the text, in practice it was usually possible to identify questionable peaks by collection, saponification, and identification of the resulting retinol isomers with the use of dioxane/hexane as the mobile phase.

pressed as a fraction of the all-trans. A given esterified isomer could also be related to that of the same isomer esterified to stearic acid. Hence, all retention times were expressable as a fraction of the retention time of all-trans retinyl stearate. These relative values are summarized in the diagram of Fig. 3.

Since 11-cis and all-trans are sometimes the only major isomers found in ocular tissues, the separation of their various esters is of particular importance. However, Fig. 3 indicates that difficulties might be encountered in resolving 11-cis retinyl linoleate from all-trans retinyl stearate, and the 11-cis linolenate from the all-trans oleate.

Unambiguous identification of the isomer present in questionable peaks was carried out by collection, saponification, and chromatography of the resulting retinols in a dioxane/hexane system capable of identifying the four major retinol isomers. This approach was used in the analysis of retinyl esters extracted from rabbit and rat pigment epithelium cells, as seen below.

Analysis of retinyl esters in ocular tissues

Rat. We have confirmed previous observations that dark-adapted rats store almost no vitamin A in their ocular tissues. Therefore we worked with the light-adapted animals. Fig. 4, a, is a chromatogram of the retinyl esters extracted from light-adapted eyes. Two peaks were present. As shown in Fig. 4,
Retinyl esters from ocular tissues

Fig. 4. Retinyl ester present in the eyes of light-adapted albino rats. a, Extract. b, Extract and all-trans retinyl stearate. c, Extract and all-trans retinyl palmitate. Conditions as in Fig. 1.

b and c, the first peak co-chromatographed with all-trans retinyl stearate, and the second with all-trans retinal palmitate. Saponification of the eluted peaks yielded only the all-trans isomer of retinol. This eliminated the possible presence of 11-cis linoleate, which was not separable from the all-trans stearate (Fig. 3). The vitamin A in the eyes of light-adapted rats therefore consists of all-trans retinyl stearate and palmitate.
Fig. 5. Retinyl ester from the pigment epithelium oil droplets of dark-adapted rabbits. a, Extract. b, Extract + 11-cis retinyl palmitate. c, Extract + all-trans retinyl palmitate. Conditions as in Fig. 1.
Fig. 6. Identification of the isomeric configuration of peak 1 retinyl ester in Fig. 5. a, Mixture of authentic 11-cis and 13-cis retinols. b, Saponified peak 1. c, Saponified peak 1 + 11-cis retinol. d, Saponified peak 1 + 13-cis retinol. Mobile phase was dioxane/hexane (5% v/v). λ, 325 nm; flow, 0.7 ml/min.
Rabbit. Unlike the rat but in common with most other animals, the rabbit pigment epithelium was found to store vitamin A in the dark-adapted condition. Further, many of the cells contained large, colorless (5 to 9 μm diameter) fluorescent oil droplets (see also ref. 19) similar to those observed in the frog but absent in most mammals. In the frog it has been reported\(^6\) that about 80% of the total vitamin A stored in the eye occurred in these oil droplets, almost exclusively in the esterified state. As judged by the Carr-Price assay, the oil droplets isolated from rabbit pigment epithelium contained 8.5 nmol of vitamin A (five dark-adapted eyes) compared with 3.8 nmol for the residual heavy tissue. The oil droplets therefore accounted for about 79% of the ocular vitamin A.

The chromatogram of the oil droplet retinyl esters in Fig. 5, a, shows two well-separated peaks. A similar profile was obtained with the esters from the heavy residues. The first peak co-chromatographed with 11-cis retinyl palmitate (Fig. 5, b), and the second with all-trans retinyl palmitate (Fig. 5, c). Collection of each individual peak yielded fractions with spectra characteristic of each isomer (in hexane the first peak had λ\(_{\text{max}}\) at 317 nm, the second at 327 nm).

In order to make an unequivocal identification of these isomers, the fractions were saponified, and the resulting alcohols analyzed by hplc with dioxane/hexane. This system was capable of distinguishing 11-cis from 13-cis retinol as shown in Fig. 6, a; the 9-cis and all-trans retinols had much longer retention times.\(^{13, 15}\) Peak 1, which co-chromatographed with 11-cis retinyl palmitate in Fig. 5, yielded a single retinol isomer, as seen in Fig. 6, b. This co-chromatographed with 11-cis retinol (Fig. 6, c) but was distinct from 13-cis retinol (Fig. 6, d). This observation (reinforced by the λ\(_{\text{max}}\) of the absorption spectrum) eliminated the possibility that peak 1 was attributable to 13-cis 18:1, which co-chromatographed with 11-cis 16:0 (Fig. 3). The ester peak co-chromatographing with all-trans retinyl palmitate in Fig. 5 was similarly shown to yield all-trans retinol on saponification. As seen in Fig. 3, there is little ambiguity in identifying all-trans retinyl palmitate, since it is separable from the esters of all other isomers.

We conclude that in common with frogs and cattle,\(^6\) 7, 18 the dark-adapted rabbit eye contains both 11-cis and all-trans retinyl esters. Both esters exist almost exclusively as palmitate. Retinol esterification in the rabbit pigment epithelium therefore is selective with regard to fatty acid utilization. In contrast, the retinyl esters stored in the liver or synthesized by retinal tissue preparations contain appreciable quantities of stearate, oleate, and other fatty acids in addition to the palmitate, which represents only 60% of the total.\(^4, 16\) This situation might be explicable if the pigment epithelium had enzyme systems or retinyl ester transport proteins with specificities that impose constraints on the ester chain length and degree of unsaturation.

REFERENCES
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