

## In vivo biosynthesis of cholesterol in the rat retina

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Received 30 August 1993; revised version received 10 October 1993

Previous reports have suggested that the rate of de novo cholesterol synthesis in the adult vertebrate retina is extremely slow. We investigated cholesterol biosynthesis in the adult rat retina in vivo, following intravitreal injection of [<sup>3</sup>H]acetate. HPLC analysis of retinal non-saponifiable lipid extracts revealed co-elution of radioactivity with endogenous cholesterol mass within 4.5 h post-injection. Incorporation of [<sup>3</sup>H]acetate into cholesterol was markedly reduced by co-injection of known inhibitors of the cholesterol pathway. In contrast to previous results with retinas from other species, no radiolabel or mass corresponded to squalene, except in lipid extracts from retinas treated with NB-598, a squalene epoxidase inhibitor. These results demonstrate, for the first time, the capacity of the adult vertebrate retina to rapidly synthesize cholesterol de novo.

Cholesterol; Lovastatin; NB-598; Lipid Metabolism; Retina; Rat

### 1. INTRODUCTION

Although cholesterol is a constituent of vertebrate retinas [1], and has been implicated in the modulation of photoreceptor function [2], its biogenic origin remains to be determined. Studies performed in vitro with isolated adult bovine [3] and frog [4,5] retinas or retinal homogenates [6] in short-term incubations (1–6 h) have shown that little, if any, cholesterol is synthesized from radiolabeled de novo precursors. Instead, the major radiolabeled non-saponifiable lipid products is squalene, with lesser and variable amounts of sterol intermediates (e.g. lanosterol). Similar results have been obtained in vivo, 1–2 h following intravitreal administration of [<sup>3</sup>H]mevalonate in the bovine eye [7]. In the frog eye, conversion of [<sup>3</sup>H]mevalonate or [<sup>3</sup>H]acetate to cholesterol (as the major radiolabeled product) takes  $\geq 1$  day [8]. Unlike bovine retina or rod outer segment membranes [9], frog retina and rod outer segment membranes contain significant amounts of squalene mass [4,5], in addition to cholesterol. Furthermore, the squalene produced from radiolabeled de novo precursors in frog retina is incorporated into the rod outer segments, where it apparently is not further metabolized [8].

We recently described [10] the production of a dysplastic-like retinal degeneration in adult rats following intravitreal injection of lovastatin, a potent inhibitor of HMG-CoA reductase (EC 1.1.1.34) [11]. In addition

to blocking cholesterol biosynthesis, this inhibitor also affects the synthesis of a variety of other biologically significant products, such as isoprenyl pyrophosphates, ubiquinones, dolichols, and isoprenylated proteins [12,13]. Due to the fact that cholesterol biogenesis is thought to be negligible in retina, the involvement of the sterol branch of the isoprenoid pathway in the etiology of the lovastatin-induced retinal degeneration was discounted [10]. However, studies concerning the de novo synthesis of cholesterol in the rat retina have not been reported, heretofore. Herein, we present the first evidence for rapid de novo synthesis of cholesterol in the rat retina in vivo, as well as the ability of known inhibitors of the cholesterol pathway to block retina isoprenoid lipid synthesis, using [<sup>3</sup>H]acetate as a radiolabeled precursor.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

Unless otherwise specified, all reagents were analytical reagent grade and obtained from Sigma. All solvents were HPLC grade and obtained from Burdick and Jackson. [<sup>3</sup>H]Acetate (Na salt, 27 Ci/mmol) was from ICN Radiochemicals. [4-<sup>14</sup>C]Cholesterol (40 mCi/mmol) and [1,5,9,14,20,24-<sup>14</sup>C]squalene (50 mCi/mmol) were from Du Pont/NEN Research Products and American Radiolabeled Chemicals, respectively. Non-radioactive standards of cholesterol, lanosterol, and squalene were from Sigma. Lathosterol (5 $\alpha$ -cholest-7-en-3 $\beta$ -ol) was from Research Plus Steroids; desmosterol (cholesta-5,24-dien-3 $\beta$ -ol) was from Steraloids, Inc. The following compounds were obtained as gifts from the sources specified: lovastatin (Dr. A.W. Alberts, Merck Sharpe & Dohme), NB-598 (Dr. M. Horie, Banyu Pharmaceutical Co.), and 5 $\alpha$ -cholest-8(14)-en-3 $\beta$ -ol (Dr. R.A. Pascal, Princeton University).

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## 2.2. Intravitreal injections

All procedures involving animals conformed to the *ARVO Resolution on the Use of Animals in Research* and the *NIH Guide for the Care and Use of Laboratory Animals*. Adult female Long-Evans pigmented rats (200–250 g), maintained for at least 1 week on cyclic lighting (12 h light, 12 h dark), were anesthetized by intraperitoneal injection with a ketamine–acetylpromazine mixture. Intravitreal injections were performed as described previously [10], using a 32-gauge needle affixed to a 25  $\mu$ l Hamilton syringe barrel. Two groups of animals (4 per group) were used. In one group, one eye of each animal received [ $^3$ H]acetate (0.25 mCi, dissolved in 7.5  $\mu$ l of 10 mM Tris-Cl, pH 7.4); the contralateral eye received a solution containing the same amount of [ $^3$ H]acetate, supplemented with lovastatin (100  $\mu$ g in 7.5  $\mu$ l 10 mM Tris-Cl, pH 7.4), prepared as the Na salt [14]. In the second group, one eye of each animal received 0.25 mCi of [ $^3$ H]acetate in 5  $\mu$ l of Me<sub>2</sub>SO, while the contralateral eye was injected with 5  $\mu$ l of Me<sub>2</sub>SO containing 0.25 mCi of [ $^3$ H]acetate plus NB-598 (50  $\mu$ g), an inhibitor of squalene-2,3-epoxidase (EC 1.14.99.7) [15]. In this group, Me<sub>2</sub>SO was used in place of Tris-Cl buffer as a vehicle, due to the limited solubility of NB-598 in aqueous buffer. After allowing 4.5 h (NB-598 group) or 6 h (lovastatin group) for precursor uptake and metabolism, the animals were sacrificed by intracardiac pentobarbital overdose, enucleated, and the neural retinas were isolated.

## 2.3. Extraction and analysis of retina nonsaponifiable lipids

Retinas were individually saponified in 1 ml of methanolic KOH (60% (w/v) KOH in 80% MeOH, 20% H<sub>2</sub>O (v/v)) for 1 h at 100°C under argon in sealed glass tubes. After cooling and addition of 2 ml of H<sub>2</sub>O, the non-saponifiable lipids were extracted with petroleum ether (2  $\times$  5 ml each); the combined extracts were washed with 1 ml of 5% (v/v) acetic acid, evaporated to dryness under argon, and redissolved in 0.20 ml of MeOH. An aliquot (20  $\mu$ l) of the extract was taken for measurement of radioactivity by liquid scintillation counting. Another aliquot (100  $\mu$ l) was taken for analysis by reverse-phase HPLC. A Waters/Millipore 600 series HPLC system was employed, equipped with either an Ultracarb 3 ODS-20 reverse-phase column (4.6  $\times$  150 mm, 3  $\mu$ m resin; Phenomenex) or a silica column (4.6  $\times$  220 mm, 5  $\mu$ m resin; Brownlee Laboratories). For reverse-phase HPLC, a mobile phase of 100% MeOH at 1 ml/min was employed; for straight-phase (silica) HPLC, the mobile phase was 1% (v/v) reagent alcohol in 99% (v/v) hexane, at a flow rate of 1 ml/min. Radioactivity

was detected with a Radiomatic Flo-One/Beta model CR flow-through liquid scintillation detector (0.5 ml flow cell), using Packard Flo-Scint III cocktail (4:1 v/v ratio, relative to mobile phase;  $^3$ H counting efficiency, 35%;  $^{14}$ C counting efficiency, 95%). Mass was detected simultaneously by UV absorbance (210 nm) with a Waters/Millipore model 481 spectrophotometer, and quantified (in comparison with a series of cholesterol standards) using a Waters/Millipore model 745B integrator. The elution positions for mass and radioactivity were calibrated using  $^{14}$ C-labeled and non-radioactive standards of cholesterol and squalene.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of lovastatin on [ $^3$ H]acetate incorporation into retina non-saponifiable lipids

Fig. 1 shows representative reverse-phase, radio-HPLC profiles obtained for rat retina non-saponifiable lipids, 6 h following intravitreal injection of [ $^3$ H]acetate, with (Fig. 1B) or without (Fig. 1A) companion lovastatin. In both cases, a major UV-absorbing component was observed with a retention time of 11.7 min. Under the chromatographic conditions employed, an authentic standard of cholesterol eluted with a retention time of 11.7 min, and was well resolved from standards of desmosterol (retention time 9.7 min), 5 $\alpha$ -cholest-8(14)-en-3 $\beta$ -ol (retention time 10.2 min), and squalene (retention time 13.7 min) (see Fig. 1C). Based upon the integrated UV absorbance values of the 'cholesterol-like' mass peaks, the cholesterol mass in the control and lovastatin-treated samples was comparable (80.4 nmol and 86.4 nmol, respectively).

In the radiochromatogram of the control sample (Fig. 1A), the major peak of radioactivity ( $\approx$  65% of total recovery) was coincident with the cholesterol-like mass peak. In addition,  $\approx$  18% of the recovered radioac-

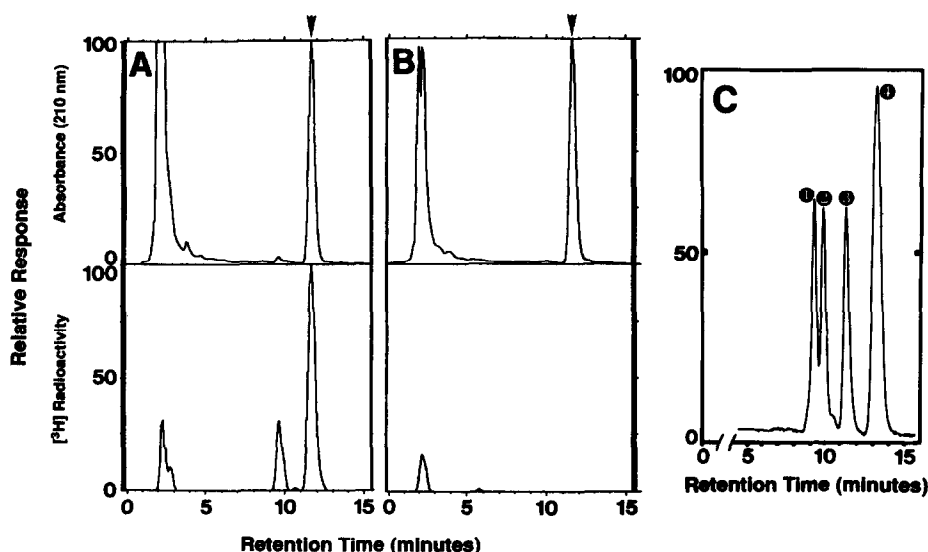


Fig. 1. Reverse-phase radio-HPLC profile of rat retina non-saponifiable lipids, obtained 6 h after intravitreal injection of [ $^3$ H]acetate (0.25 mCi/eye) in the absence (A) or presence (B) of lovastatin (Na salt, 100  $\mu$ g). Upper panels (A,B), UV absorbance (210 nm), normalized to give full-scale response for cholesterol (arrowheads); lower panels,  $^3$ H radioactivity (total recovered radioactivity: A, 8,170 dpm; B, 790 dpm) normalized to same full-scale response. (C) Elution profile of authentic standards of (1) desmosterol, (2) 5 $\alpha$ -cholest-8(14)-en-3 $\beta$ -ol, (3) cholesterol, and (4) squalene.

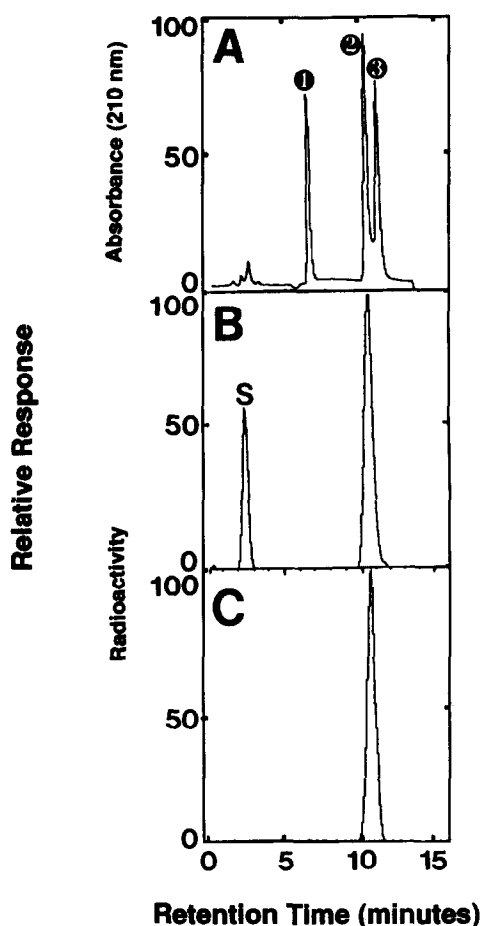


Fig. 2. Straight-phase (silica) radio-HPLC elution profiles. (A) UV absorbance (210 nm) of authentic standards of (1) lanosterol, (2) cholesterol, and (3) lathosterol. (B)  $^{14}\text{C}$ -labeled standards of squalene (S) and cholesterol (full scale, 500 dpm). (C) Cholesterol-like,  $^3\text{H}$ -labeled material (see Fig. 1A) obtained by reverse-phase HPLC of control rat retina non-saponifiable lipids (full scale, 2,500 dpm).

tivity eluted with a retention time of 9.7 min, consistent with the chromatographic behavior of desmosterol or a related  $\text{C}_{27}$   $3\beta$ -monohydroxy sterol. In contrast, the lovastatin-treated sample (Fig. 1B) exhibited no radiolabeled components corresponding to cholesterol or known intermediates in the sterol biosynthetic pathway. Virtually all of the recovered radioactivity (which corresponded to  $\leq 10\%$  of the control value) consisted of very polar material of unknown identity (retention time 2.2 min); similar radiolabeled material, representing  $\approx 14\%$  of the recovered radioactivity, was also observed in the control sample. These results are consistent with the expected inhibition of HMG-CoA reductase by lovastatin [11]. Notably, in contrast to results obtained in previous studies with vertebrate retinas [3–8], neither the control nor the lovastatin-treated sample exhibited detectable mass or radioactivity corresponding to squalene.

A portion of the cholesterol-like material from the control sample was collected from the reverse-phase

column (11.1–12.7 min), evaporated to dryness, supplemented with internal standards of  $^{14}\text{C}$ -labeled cholesterol (3,200 dpm) and squalene (1,200 dpm), plus non-radiolabeled lanosterol (10  $\mu\text{g}$ ) and lathosterol (20  $\mu\text{g}$ ), and then analyzed by straight-phase HPLC. The results are shown in Fig. 2. The  $^3\text{H}$  radioactivity derived biosynthetically from rat retina (Fig. 2C) eluted quantitatively as a homogeneous peak with a retention time of 10.5 min, which corresponded exactly to the elution time of both the internal [ $^{14}\text{C}$ ]cholesterol standard (Fig. 2B) and the cholesterol mass peak (Fig. 2A). Under the chromatographic conditions employed, cholesterol was well resolved from squalene (retention time 2.5 min), lanosterol (retention time 6.9), and lathosterol (retention time 11.5 min).

### 3.2. Effect of NB-598 on [ $^3\text{H}$ ]acetate incorporation into retina non-saponifiable lipids

In a separate experiment, rats were injected intravitreally with [ $^3\text{H}$ ]acetate, with or without the squalene epoxidase inhibitor, NB-598, and the retinas were removed 4.5 h later. Representative reverse-phase radio-HPLC chromatograms of the corresponding non-saponifiable lipid extracts are shown in Fig. 3. In the course of this particular chromatographic analysis, standards of cholesterol and squalene exhibited retention times of  $\approx 14.0$  and 15.2 min, respectively ( $\pm 0.1$  min). In the radiochromatogram of the control sample (Fig. 3A), the most prominent peak of radioactivity ( $\approx 54\%$  of total recovery) was coincident with endogenous mass, having the chromatographic properties of cholesterol; again, no radiolabel or mass corresponded to squalene. In the chromatogram of the sample obtained from an eye that was co-injected with [ $^3\text{H}$ ]acetate and NB-598 (Fig. 3B), not only did the most prominent peak of radioactivity correspond to squalene ( $\approx 42\%$  of total recovery), but there was detectable mass coincident with the squalene standard. These results are consistent with the expected effects of a squalene epoxidase inhibitor [15]. Some radioactivity ( $\approx 15\%$  of total recovery) was also detected coincident with the cholesterol mass peak; however, the relative amount of radiolabel corresponding to cholesterol was reduced almost 4-fold, relative to the control. Hence, either the conditions were not optimal for complete squalene epoxidase inhibition, or some [ $^3\text{H}$ ]acetate uptake and conversion to sterols took place prior to inhibition by NB-598. In both chromatograms, a cluster of radiolabeled components was observed with retention times in the range of  $\approx 10.5$ –12 min; these peaks were markedly reduced in the NB-598-treated sample, relative to the control, suggesting that they may correspond to sterol intermediates in the cholesterol pathway. However, these components were not characterized further.

### 3.3. Cholesterol synthesis in the vertebrate retina

Taken together, these results demonstrate that rat

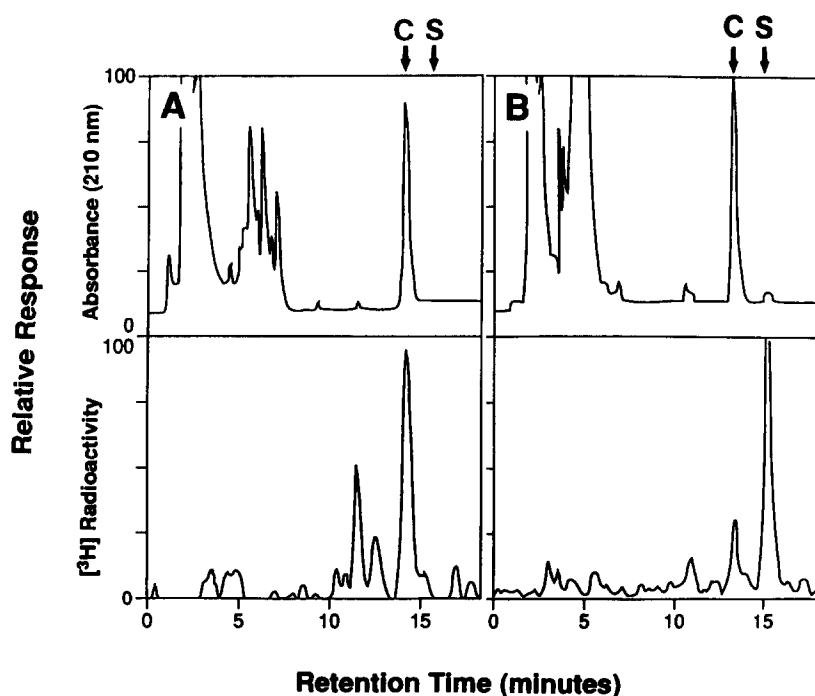


Fig. 3. Reverse-phase radio-HPLC profile of rat retina non-saponifiable lipids, obtained 4.5 h after intravitreal injection of [ $^3\text{H}$ ]acetate (0.25 mCi/eye) in the absence (A) or presence (B) of NB-598 (50  $\mu\text{g}$ ). Upper panels, UV absorbance (210 nm); lower panels,  $^3\text{H}$  radioactivity (total recovered radioactivity: A, 12,100 dpm; B, 17,280 dpm). Arrows denote elution positions of authentic cholesterol (C) and squalene (S) standards, under the conditions employed.

retina is capable of relatively rapid *de novo* synthesis of cholesterol *in vivo*. In contrast, previous studies with other species, most of which were performed with either isolated retinas *in vitro* [3–5] or retinal homogenates [6], have shown minimal *de novo* synthesis of cholesterol, with attendant accumulation of squalene and sterol intermediates. It is unlikely that this discrepancy is simply a function of *in vitro* vs. *in vivo* factors, since such sterol precursor accumulation has also been observed *in vivo* in bovine [7] and frog retinas [8]. In frog retina, appreciable synthesis of cholesterol requires several hours-to-days. Considering the obvious merits of the rat as an experimental animal for comparative *in vivo* and *in vitro* studies of retina metabolism [16,17], the rat is recommended as the animal of choice for further investigations concerning cholesterol synthesis, intracellular transport, turnover, and function in the vertebrate retina.

### 3.4. Lovastatin-induced retinal degeneration

The selective inhibition of the sterol pathway afforded by NB-598, as opposed to the generalized inhibition of isoprenoid metabolism produced with lovastatin, provides the means to specifically evaluate the involvement of cholesterol metabolism in the lovastatin-induced retinal degeneration [10]. Preliminary results in our laboratories (unpublished) indicate that intravitreal NB-598 does *not* produce a retinal degeneration, under conditions where cholesterol synthesis is inhibited by

80–100%. Currently, we are pursuing more detailed studies of the dose dependence and persistence of the inhibitory activity of intravitreal lovastatin and NB-598 with regard to their histological and functional consequences on the rat retina.

**Acknowledgements:** The technical assistance of David A. Saperstein, M.D., and Julia Harris, and the generous gifts of lovastatin (Dr. A.W. Alberts, Merck Sharpe & Dohme), NB-598 (Dr. M. Horie, Banyu Pharmaceutical Co.), and  $5\alpha$ -cholest-8(14)-en-3 $\beta$ -ol (Dr. R.A. Pascal, Princeton University) are gratefully acknowledged. This study was supported by USPHS Grants EY07361 (S.J.F., R.F., R.K.K.), EY04554 and EY02520 (L.M.R.), by a grant from the Knights Templar Eye Foundation Inc. (S.J.P.), and by unrestricted departmental grants from Research to Prevent Blindness Inc. S.J.F. is a Research to Prevent Blindness James S. Adams Scholar.

### REFERENCES

- [1] Fliesler, S.J. and Anderson, R.E. (1983) *Progr. Lipid Res.* 22, 79–131.
- [2] Boesze-Battaglia, K. and Albert, A.D. (1990) *J. Biol. Chem.* 265, 20727–20730.
- [3] Fliesler, S.J. and Schroepfer Jr., G.J., (1986) *J. Neurochem.* 46, 448–460.
- [4] Keller, R.K., Fliesler, S.J. and Nellis, S.W. (1988) *J. Biol. Chem.* 263, 2250–2254.
- [5] Keller, R.K. and Fliesler, S.J. (1990) *J. Biol. Chem.* 265, 13709–13712.
- [6] Fliesler, S.J. and Schroepfer Jr., G.J. (1983) *J. Biol. Chem.* 258, 15062–15070.
- [7] Fliesler, S.J. (1979) Ph.D. Thesis, Rice University, Houston, TX.
- [8] Keller, R.K., Florman, R. and Fliesler, S.J. (1993) *Invest. Ophthalmol. Vis. Sci. (ARVO Abstr.)* 34, 1130.

- [9] Fliesler, S.J. and Schroepfer Jr., G.J. (1982) *Biochim. Biophys. Acta* 711, 138–148.
- [10] Pittler, S.J., Fliesler, S.J. and Rapp, L.M. (1992) *Exp. Eye Res.* 54, 149–152.
- [11] Alberts, A.W., Chen, J., Kuron, G., et al. (1980) *Proc. Natl. Acad. Sci. USA* 77, 3957–3961.
- [12] Schroepfer Jr., G.J. (1981) *Annu. Rev. Biochem.* 50, 585–621.
- [13] Sinensky, M. and Lutz, R.J. (1992) *BioEssays* 14, 25–31.
- [14] Kita, T., Brown, M.S. and Goldstein, J.L. (1980) *J. Clin. Invest.* 66, 1094–1100.
- [15] Horie, M., Tsuchiya, Y., Hayashi, M., et al. (1990) *J. Biol. Chem.* 265, 18075–18078.
- [16] O'Brien, P.J. (1993) in: *Methods in Neurosciences*, vol. 15, (Hargrave, P.A. ed) pp. 75–85, Academic Press, Orlando, FL.
- [17] Fliesler, S.J. (1993) in: *Methods in Neurosciences*, vol. 15, (Hargrave, P.A. ed) pp. 86–107, Academic Press, Orlando, FL.