In the vertebrate retina, a number of proteins involved in signal transduction are known to be N-terminal acylated with the unusual 14 carbon fatty acids 14:1-9 and 14:2-6. We have explored possible pathways for producing these fatty acids in the frog retina by incubation in vitro with candidate precursor fatty acids bearing radiolabels, including [3H]14:0, [3H]18:1n-9, [3H]18:2n-6, and [3H]18:3n-3. Rod outer segments were prepared from the radiolabeled retinas for analysis of protein-linked fatty acids, and total lipids were extracted from the remaining retinal pellet. Following saponification of extracted lipids, fatty acid phenacyl esters were prepared and analyzed by high pressure liquid chromatography (HPLC) with detection by continuous scintillation counting. Transducin, whose α-subunit (Gtα) is known to bear N-terminal acyl chains, was extracted from the rod outer segments and subjected to SDS-polyacrylamide gel electrophoresis and fluorography to detect radiolabeled proteins. Gtα was also subjected to methanalysis, and the resulting fatty acyl methyl esters were analyzed by HPLC. The identities of HPLC peaks coinciding with unsaturated species of both phenacyl esters and methyl esters were confirmed by reanalyzing them after catalytic hydrogenation. The results showed that 14:1-9 can be derived in the retina from 18:1-9 and 14:2-6 from 18:2-6, most likely by two rounds of β-oxidation, but that neither is produced in detectable amounts from 14:0. Retroconversion of unsaturated 18 carbon fatty acids to the corresponding 14 carbon species showed specificity, in that 18:3-3 was not converted to 14 carbon fatty acids in detectable amounts. Myristic acid (14:0), 14:1n-9 has been shown to play an important role in the function of these proteins (Jones et al., 1990; Linder et al., 1991; Yonemoto et al., 1993; Wedegaertner et al., 1995). In vertebrate retinas, the types of N-terminal fatty acids for proteins involved in signal transduction are strikingly different from those found in other tissues. These proteins, which include the α-subunit of the G protein transducin (Gtα), guanylyl cyclase-activating protein (GCAP) (Palczewski et al., 1994), recoverin (Dizhoor et al., 1992), and the catalytic subunit of cAMP-dependent protein kinase (J ohnson et al., 1994), are heterogeneously acylated with frequent occurrence of 14:1-9 and 14:2-6 in addition to 14:0 and 12:0. This unusual pattern appears to reflect unusual pathways for synthesizing and utilizing fatty acids for N-terminal acylation in the retina rather than specific characteristics of the acylated proteins. The ubiquitous protein kinase is exclusively modified with 14:0 in the brain and heart (Carr et al., 1982; Johnson et al., 1994) but in the retina shows the same heterogeneous pattern of fatty acylation as Gtα. Indeed, although 12:0 and 14:0 are found in animal tissues, 14:1-9 and 14:2-6 are quite rare and have been found in abundance only in marine mammals (14:1-9) (Markley, 1960) and the Asian plant Evodia rutaceaarp (14:2-6) (Kuron o et al., 1972). It is conceivable that the two unsaturated fatty acids could arise from the desaturation of 14:0. However, previously described Δ4-desaturase enzymes prefer long chain polyunsaturated fatty acids as substrates (Numa et al., 1984), and Δ4 desaturases have only been found in a few cell types, such as testicular Sertoli (Oulhaj et al., 1992) and brain glial cells (Cook et al., 1991). Alternatively, these unusual 14-carbon fatty acids might arise by retroconversion from longer chain fatty acids incubated with 18:3-3. Thus, retroconversion by limited β-oxidation of longer chain unsaturated fatty acids appears to be the most likely metabolic source of the unusual fatty acids found on the N termini of signal transducing proteins in the retina.

A number of proteins of both eukaryotic and viral origin are modified by fatty acylation through an amide linkage to N-terminal glycine residues (reviewed by James and Olson (1990), McIlhinney (1990), Towler et al. (1988), Schlesinger (1993), and Gordon et al. (1991)). This modification, which in the great majority of cases studied involves the saturated 14 carbon fatty acid myristate (14:0), has been shown to play an important role in the function of these proteins (Jones et al., 1990; Linder et al., 1991; Yonemoto et al., 1993; Wedegaertner et al., 1995). In vertebrate retinas, the types of N-terminal fatty acids for proteins involved in signal transduction are strikingly different from those found in other tissues. These proteins, which include the α-subunit of the G protein transducin (Gtα), guanylyl cyclase-activating protein (GCAP) (Palczewski et al., 1994), recoverin (Dizhoor et al., 1992), and the catalytic subunit of cAMP-dependent protein kinase (Johnson et al., 1994), are heterogeneously acylated with frequent occurrence of 14:1-9 and 14:2-6 in addition to 14:0 and 12:0. This unusual pattern appears to reflect unusual pathways for synthesizing and utilizing fatty acids for N-terminal acylation in the retina rather than specific characteristics of the acylated proteins. The ubiquitous protein kinase is exclusively modified with 14:0 in the brain and heart (Carr et al., 1982; Johnson et al., 1994) but in the retina shows the same heterogeneous pattern of fatty acylation as Gtα. Indeed, although 12:0 and 14:0 are found in animal tissues, 14:1-9 and 14:2-6 are quite rare and have been found in abundance only in marine mammals (14:1-9) (Markley, 1960) and the Asian plant Evodia rutaceaarp (14:2-6) (Kuron o et al., 1972). It is conceivable that the two unsaturated fatty acids could arise from the desaturation of 14:0. However, previously described Δ4-desaturase enzymes prefer long chain polyunsaturated fatty acids as substrates (Numa et al., 1984), and Δ4 desaturases have only been found in a few cell types, such as testicular Sertoli (Oulhaj et al., 1992) and brain glial cells (Cook et al., 1991). Alternatively, these unusual 14-carbon fatty acids might arise by retroconversion from longer chain fatty acids.
acids through partial β-oxidation. For example, 18:1n-9 (oleic acid) or 18:2n-6 (linoleic acid) might be converted by this route to 14:1n-9 and 14:2n-6, respectively. Indeed, it has been hypothesized that 14:2n-6 is generated within the photoreceptor cell in this manner (Hansen, 1993; Wang and Anderson, 1993). Such partial β-oxidation is characteristic of peroxisomal metabolism in contrast to mitochondrial β-oxidation, which favors complete degradation to acetyl-CoA (Schulz, 1991). Retroconversion pathways of this kind have been demonstrated to convert 13-hydroxy-9,11-octadecadienoic acid (13-OH, 18:2n-6) to hydroxy-5,7-tetradecadienoic acid (13-OH, 14:2n-6) in lymphotoxins (Hadjuagapiono et al., 1990) and to 18:2n-6 to 14:2n-6 in rat liver peroxisomes (Baykoucheva et al. 1994). In the retina, a similar retroconversion pathway was found to convert 22:5n-6 to 20:5n-3 (Wang and Anderson, 1993). We describe here experiments designed to determine if retroconversion pathways can produce 14:1n-9 and 14:2n-6 in the frog retina and if they are used for N-terminal fatty acylation of Gt.**

**EXPERIMENTAL PROCEDURES**

**Animals—Adult frogs (Rana pipiens) were purchased from J. M. Hazen (Alburg, VT). Frogs were housed under a constant dinural cycle of 14 h light, 10 h light. All animal care and use was conducted in accordance with guidelines set by the Association for Research in Vision and Ophthalmology for the use of Animals in Research and the NIH Guide for the Care and Use of Animals in Research. The experimental protocol was reviewed and approved by the Animal Protocol Review Committee of Baylor College of Medicine (Houston, TX). [3H]Fatty Acids—[9,10-3H]18:0 (myristic acid) (31 Ci/mmol) and [9,10,12,13-3H]18:1n-9 (oleic acid) (10 Ci/mmol) were purchased from DuPont NEN. [9,10,12,13,15,16-3H]18:2n-6 (linoleic acid) (90 Ci/mmol) and [9,10,12,13,15,16-3H]18:3n-3 (linolenic acid) (120 Ci/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Each fatty acid was examined for purity as outlined in the section "Fatty Acid Phenacyl Ester Preparation and HPLC Analysis." The fatty acid mixtures were found to be free of interfering contaminants. The [3H]fatty acid, 1 mCi total for [3H]14:0 or [3H]18:1n-9 and 120 μCi total for [3H]18:2n-6 or [3H]18:3n-3, was suspended by sonication in a 200-μl aliquot of fatty acid-free (<0.003%) bovine serum albumin (Sigma) in 50 mM NaHCO3, such that the final fatty acid/bovine serum albumin molar ratio was 2:1.

**Preparation of Frog Retinas—All dissection procedures described here were performed under dim red lights. Desemembrated frogs (10 g) were killed by decapitation followed by immediate pithing of the spinal column. Eyes were enucleated and placed in room temperature Krebs-Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.17 mM KH2PO4, 1.17 mM MgSO4, 5.6 mM d-glucose, 35 mM NaHCO3, and 1.0 mM EDTA, pH 7.4) and then converted to eyecups through removal of the cornea, lens, and vitreous body. Retinas were dissected from the eyecups with jeweler’s forceps and then stored in ice-cold Krebs-Ringer buffer.

**Retina in Vitro Incubation—All ensuing incubation procedures were performed under dim room lighting (50–100 lux). Dissected frog retinas (24–32) were placed in a flask containing 3.8 ml of oxygenated (1 h with O2/CO2 95:5) Krebs-Ringer buffer supplemented with 2.5 mM CaCl2 and antibiotic mixture (final concentrations, 20 units/ml penicillin G, 20 units/ml streptomycin sulfate, and 50 ng/ml Fungizone) with centrifugation at 27,000 rpm for 10 min, and then stored in ice-cold Krebs-Ringer buffer.

**Lipid Extraction and Separation of Lipid Species—**Lipid extraction and separation of lipid species were performed by the method of Ohguro et al. (1970), who isolated lipids by chloroform extraction, followed by hexane or ether extraction, and then further purified and separated by TLC. Lipids were pooled on HPLC cartridges (Kupke and Zeugner, 1978) on a silica gel 60 TLC plate (EM, Germany) and eluted with 80% CH3OH/CHCl3. Following centrifugation, the CHCl3 layers were pooled, total lipid phosphorus was estimated by the method of Ross et al. (1970), and total radioactivity was determined by scintillation counting. An equal portion of each extract was separated into phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine), free fatty acids, and triglycerides by TLC (Kupke and Zeugner, 1978) on a silica gel 60 TLC plate (EM Science), with development up one-third of the plate with CHCl3/MeOH/H2O (65:30:5, ν/v/v) and complete development with hexane/EtOH/H2O (80:20:15, ν/v/v). For analysis of total radioactivity in each class, positions were located by 1,2,4-trinitrobenzene and illuminating with UV and then scraped and analyzed as described below.

**Fatty Acid Phenacyl Ester Preparation and HPLC Analysis—**Total lipids extracts or isolated lipid classes were saponified for 45 min at 100°C in 2% KOH/EtOH (ν/v), diluted with H2O, acidified with 12 N HCl, and extracted three times with hexane. Free fatty acid extracts were used to prepare FAPEs according to the method of Wood and Lee (1983) as modified by Chen and Anderson (1993a). FAPEs were separat on HPLC using a Supelco (Bellefonte, PA) Supelcosil LC-18 column (25 cm × 4.6 mm I.D.) with elution (2 ml/min) by a linear gradient of CH3CN/H2O starting at 80:20 (ν/v), increasing to 92:8 in 45 min, holding at 92:8 for 10 min, and returning to 80:20 in 5 min. Elution of FAPEs was monitored by UV absorbance at 242 nm. Radioactivity profiles were obtained with an on-line continuous scintillation counter (Flio-One, Radioactive Flow Detector A-200, Radiomatic, Tampa, FL) and the scintillation fluid was added to the instrument’s storage tank at a 2.5:1 (ν/v) ratio with the column eluant. Preliminary identification of FAPE peaks was based on retention times obtained for FAPEs prepared from radiolabeled and nonradiolabeled fatty acid standards (Sigma). Fatty acid standards of 14:1n-9, 14:2n-6, and 1,4,8-C14:2n-6 were generously provided by Dr. Howard Sprecher (Department of Medical Biochemistry, Ohio State University).

**Analysis of FAPEs by Calcium Hypoiodination—**Individual FAPE peaks were collected and extracted three times with hexane, followed by saponification with 2% KOH/EtOH (ν/v), as described above. Free fatty acids were solubilized in EtOH/hexane (2:1) and bubbled vigorously with hydrogen (20 min) in the presence of ~10 mg of PtO2 (Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH) catalyst. Hypoiodination was performed by addition of 0.1 ml of 12% (w/v) 1.5 ml of CHCl3. Following centrifugation, the CHCl3 layers were pooled, total lipid phosphorus was estimated by the method of Ross et al. (1970), and total radioactivity was determined by scintillation counting. An equal portion of each extract was separated into phospholipids, free fatty acids, and triglycerides by TLC (Kupke and Zeugner, 1978) on a silica gel 60 TLC plate (EM Science), with development up one-third of the plate with CHCl3/MeOH/H2O (65:30:5, ν/v/v) and complete development with hexane/EtOH/H2O (80:20:15, ν/v/v). For analysis of total radioactivity in each class, positions were located by 1,2,4-trinitrobenzene and illuminating with UV and then scraped and analyzed as described below.

**Isolation of Transducin—**Transducin was isolated using a modification of the procedures of Ohguro et al. (1990) and Umberger et al. (1992). ROS were homogenized at 4°C in isotonic wash buffer (100 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000 × g for 30 min. The isotonic buffer wash was repeated once, and the ROS pellet was homogenized at 4°C in hypotonic wash buffer (5 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000 × g for 30 min. The ROS pellet was homogenized at 4°C in hypotonic wash buffer containing 200 μM GTP-γS and centrifuged at 100,000 × g for 30 min. The hypotonic buffer wash with 200 μM GTP-γS was repeated once, and the transducin subunits (Gαi) were recovered in the GTP-γS wash supernatants. The Gαi subunits from the supernatants for the GTP-γS washes were made 16% (w/v) in trichloroacetic acid (Fisher) and centrifuged at 27,000 × g for 30 min. The protein pellet was solubilized in strongly reducing sample application buffer (20% [v/v] sucrose, 2% [w/v] SDS, 50 mM Na2CO3, pH 9, 50 mM dithiothreitol, 0.3 mM 2-mercaptoethanol, and 0.15 mM bromphenol blue) and incubated 16 h at 25°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using 12% (w/v) polyacrylamide gels. Gels were stained with Coomassie Blue R-250, destained for 2–3 days with MeOH/HAOAc/H2O (1:1:8, v/v,v), and then dried and analyzed.
After washing for 1–2 h in 10% (v/v) HOAc, the gels were treated with DuPont NEN EN3HANCE following the manufacturer's instructions. After vacuum drying onto filter paper, the treated gels were exposed to autoradiography X-ray film at 80°C for 4–6 weeks.

To test for resistance of radiolabel of proteins to hydrolysis by hydroxylamine (NH2OH) as evidence of amide linkage, SDS-PAGE was repeated and gels were treated with NH2OH using a modification of the methods of Buss et al. (1987) and Olson et al. (1985). The gels were fixed (30 min) in isopropanol/H2O/HOAc (25:65:10, v/v/v), washed (45 min) with H2O, soaked (16 h) in 1 M NH2OH · HCl (pH 6.7), and washed (6 h) with isopropanol/H2O/HOAc (10:80:10, v/v/v). Gels were stained, destained, treated with EN3HANCE™, dried, and autoradiographed. To determine the efficiency of thioester-linked fatty acid removal by NH2OH, we performed the procedure on rhodopsin, a protein known to carry two thioester linked 16:0 groups (Papac et al., 1992). Using previously described procedures, frog retinas were incubated in vitro with 150 μCi of [9,10(14N)-3H]16:0 (American Radiolabeled Chemicals Inc., St. Louis, MO), and ROS were prepared, stripped of transducin, and subjected to SDS-PAGE using low reducing conditions (1 h of incubation in sample application buffer containing 2 mM dithiothreitol and 15 μM 2-mercaptoethanol). Gel slices (n = 4) containing rhodopsin (~50 μg, estimated by BCA* protein assay kit, Pierce) were saponified in 1 M NaOH at 37°C for 2 h and acidified with 12 N HCl, and radioactivity was determined by counting gel slice and hydrolysate together (BCS mixture/hydrolysate, 15:1, v/v). Identical gels were sub-

**Fig. 1. Incubation with [9,10-3H]14:0.** A, HPLC elution profile of FAME radioactivity for total lipids extracted from frog retinas incubated with 1 mCi of [3H]14:0. The y axis ([3H] counts/6-s interval) is shown at a 10× reduction of the original scale; the maximum for [3H]14:0 peak was 41,900. B, Coomassie Blue-stained electrophoretic gel (a) and corresponding fluorogram (b) for transducin (Gαβγ) isolated from frog retinas labeled with 1 mCi of [3H]14:0. Fluorography was performed for 28 days. Gα and Gβ are the α- and β-subunits of transducin, respectively. The Gγ (8 kDa) was run off the gel. C, HPLC elution profiles of FAME radioactivity for Gα and Gβ (control) isolated from frog retinas labeled with 1 mCi of [3H]14:0. FAMEs were released from the SDS-PAGE-purified Gα and Gβ by acidic methanolysis.
FIG. 2

Biosynthesis of Fatty Acids Found on Photoreceptor Proteins
ected to treatment with 1 mM HClO₄ as described above; rhodopsin was excised, saponified, and counted. NH₄OH treatment removed 84 ± 2% of the radiolabel from this protein.

Fatty Acid Analysis of Transducin—Samples of transducin (n = 3) were subjected to SDS-PAGE and amounts of Gₐ and Gₐβ were estimated by scanning the gel photographs. The relative amount of Gₐ and Gₐβ was estimated by using a densitometer (Personal Densitometer 120, Molecular Dynamics Inc., Sunnyvale, CA) using a linear proportion comparison with a known amount of carbonic anhydrase (29 kDa) run on the same gel. The amounts of Gₐ in each were estimated to be 18 ± 4 (14:0), 24 ± 4 (18:1n-9), 24 ± 2 (18:2n-6), and 8 ± 1 µg (18:3n-3), with corresponding Gₐβ at 13 ± 2, 16 ± 3, 15 ± 2, and 5 ± 1 µg, respectively. Gel slices containing Gₐ and Gₐβ were washed with MeOH:H₂O:HOAc (50:40:1, v/v/v) (24 h), 50% MeOH (24 h), and 100% MeOH (2 h). After the addition of 100 nmol 16:1n-9 and 17:0 as carrier, the gel slices were hydrolyzed in 2 v HCl 83% MeOH for 8 h at 98 °C under argon gas. The hydrolysate was extracted three times with hexane, and the fatty acid methyl esters (FAMEs) were chromatographed on HPLC with UV absorbance being monitored at 192 nm (Avedano et al., 1983). The identities of unsaturated FAMEs were confirmed by catalytic hydrogenation.

RESULTS

Incubation with [9,10-3H]14:0—HPLC of FAPEs derived from the total retina lipid pool after incubation with [3H]14:0 (Fig. 1A) revealed only chain elongation to [3H]16:0 (−11% of [3H]14:0) and [3H]18:0 (−7%). A minor peak eluting at 14 min had the identical retention time as a contaminant that was present in the commercial stock of [3H]14:0, and therefore is unlikely to represent a metabolic product of 14:0.

The Coomassie staining pattern (Fig. 1B, a) and fluorogram (Fig. 1B, b) showed intense radiolabeling at a migration position in SDS-PAGE aligning precisely with Gₐ (39 kDa), and there was no detectable radiolabeling of Gₐβ (36 kDa) or any other proteins. HPLC of methanolation products (Fig. 1C) confirmed that the radiolabel on Gₐ was 14:0 (481 ± 74 counts/6 s/10 µg), along with some minor peaks of uncertain identity. A comparatively insignificant amount of labeled 14:0 was associated with Gₐβ (18 ± 11 counts/6 s/10 µg). Incubation with [9,10-3H]18:1n-9—HPLC of FAPEs derived from the total retina lipid pool after incubation with [3H]18:1n-9 (Fig. 2A) demonstrated only retroconversion to [3H]14:1n-9 (−2% of [3H]18:1n-9) and [3H]16:1n-9 (−4%). HPLC of the retroconversion products after catalytic hydrogenation (Fig. 2A) confirmed their identities by showing an appropriate shift in retention time to that of 14:0 and 16:0, respectively. A trace amount of 14:0 was also produced during the hydrogenation of 16:1n-9.

The Coomassie staining pattern (Fig. 2B, a) and fluorogram (Fig. 2B, b) revealed faint radiolabeling concentrated at a migration position in SDS-PAGE aligning precisely with Gₐ (39 kDa), with some diffuse radiolabeling extending into the region for Gₐβ (36 kDa). Another area of diffuse radiolabeling was observed to be located at a migration position aligning with a faint doublet protein band above 84 kDa. HPLC of methanolysis products (Fig. 2C) confirmed the radiolabel on Gₐ was a mixture of 14:1n-9 (108 ± 16 counts/6 s/10 µg) and 18:1n-9 (51 ± 6 counts/6 s/10 µg), whereas the radiolabel seen in the region for Gₐβ was only 18:1n-9 (35 ± 9 counts/6 s/10 µg). HPLC of the Gₐ methanolysis products after simultaneous collection and catalytic hydrogenation (Fig. 2C) confirmed their identities by showing only two peaks with appropriate retention times for 14:0 and 18:0 within the predicted proportions (14:0 (63%) and 18:0 (37%)).

Incubation with [9,10,12,13-3H]18:2n-6—HPLC of FAPEs derived from the total retina lipid pool after incubation with [9,10,12,13-3H]18:2n-6 (Fig. 3A) revealed retroconversion to [3H]14:2n-6 (−2% of [3H]18:2n-6) and [3H]16:2n-6 (−1%), along with desaturation and elongation to [3H]18:3n-6, [3H]20:3n-6, and [3H]20:4n-6 (8, 3, and 7%, respectively). HPLC of the retroconversion products after catalytic hydrogenation (Fig. 3A) confirmed their identity by showing an appropriate shift in retention time to that of 14:0 and 16:0, respectively. A small amount of 18:0 was also produced during the hydrogenation of 16:2n-6, possibly arising from contaminating 18:3n-6. Identities of all desaturation and elongation products were also confirmed by catalytic hydrogenation (results not shown).

The Coomassie staining pattern (Fig. 3B, a) and fluorogram (Fig. 3B, b) revealed moderate radiolabeling at a migration position in SDS-PAGE aligning precisely with Gₐ (39 kDa), with faint radiolabeling also seen aligning with Gₐβ (36 kDa). No other areas of radiolabeling were observed in the fluorogram. HPLC of methanolation products (Fig. 3C) revealed that the radiolabel on Gₐ was only 14:2n-6 (101 ± 8 counts/6 s/10 µg), and no detectable radiolabel was associated with Gₐβ. Catalytic hydrogenation and HPLC of the methanolysis product for Gₐ confirmed its identity by showing an appropriate shift in retention time to that of 14:0.

Incubation with [9,10,12,13,15,16-3H]18:3n-3—HPLC of FAPEs derived from the total retina lipid pool after incubation with [9,10,12,13,15,16-3H]18:3n-3 (Fig. 4A) revealed the absence of candidate peaks for the anticipated 14:3n-3 or 16:3n-3 retroconversion products. Desaturation and elongation products were observed in the form of [3H]18:4n-3, [3H]20:5n-3, and [3H]22:5n-3 (−12, 8, and 7% of [3H]18:3n-3, respectively). HPLC of the 18:4n-3 desaturation product after catalytic hydrogenation (Fig. 4A) confirmed its identity by showing an appropriate shift in retention time to that of 18:0. Likewise, identities of the other desaturation and elongation products were confirmed by catalytic hydrogenation (results not shown). Hydrogenation of the prominent peak at 18 min produced a product with a retention time between that of 18:0 and 20:0 (result not shown), making its identification inconclusive.

The Coomassie staining pattern (Fig. 4B, a) and fluorogram (Fig. 4B, b) showed no radiolabeling corresponding with Gₐ (39 kDa), Gₐβ (36 kDa), or any other protein observed on SDS-PAGE. HPLC of methanolysis products (Fig. 4C) revealed that no detectable radiolabel was associated with either Gₐ or Gₐβ, consistent with the fluorogram results.

Hydroxylamine Treatment of Radiolabeled Transducin—Shown in Fig. 5 are the Coomassie staining patterns and fluorograms for gels containing transducin from the [3H]14:0, [3H]18:1n-9, and [3H]18:2n-6 incubations, after treatment with 1 mM hydroxylamine. The radiolabelings on Gₐ (39 kDa), representative of [3H]14:0, [3H]14:1n-9/[3H]18:1n-9, and [3H]14:2n-6, respectively, all showed hydroxylamine resistance. Complete disappearance of radiolabeling was observed for Gₐβ (36 kDa) and all other proteins.
Fig. 3. **Incubation with [9,10,12,13-^3H]18:2n-6.** A, HPLC elution profile of FAPE radioactivity for total lipids from frog retinas incubated with 120 μCi of [^3H]18:2n-6. The y axis ([^3H] counts/6-s interval) of the radioactivity profile is shown at a 10× reduction of the original scale; the maximum for the[^3H]18:2n-6 peak was at 32,946. Also shown are the HPLC elution profiles of FAPE radioactivity for the 14:2n-6 and 16:2n-6 after...
FIG. 4. Incubation with [9,10,12,13,15,16-3H]18:3n-3. A, HPLC elution profile of FAPE radioactivity for total lipids from frog retinas incubated with 120 μCi of [3H]18:3n-3. The y axis ([3H] counts/6-s interval) of the radioactivity profile is shown at a 4× reduction of the original scale; the maximum for the [3H]18:3n-3 peak was at 7086. Also shown are the HPLC elution profiles of FAPE radioactivity for the 18:4n-3 after being subjected to catalytic hydrogenation. B, Coomassie Blue-stained electrophoretic gel (a) and corresponding fluorogram (b) for transducin (G_{αβγ}) isolated from frog retinas labeled with 120 μCi of [3H]18:3n-3. Fluorography was performed for 35 days. G_{α} and G_{β} are the α- and β-subunits of transducin, respectively. G_{γ} (8 kDa) was run off the gel. C, HPLC elution profiles of FAME radioactivity for G_{α} and G_{β}, (control) isolated from frog retinas labeled with 120 μCi of [3H]18:3n-3. FAMEs were released from SDS-PAGE-purified G_{α} and G_{β} by acidic methanolysis.
active in other tissues. However, this does not mean 14:1 myristoyltransferase, levels of available 14:1 (Chen and Anderson, 1993a; Bartley 14:2 -6 in the total lipid pool of the frog and bovine retina n
n
CoAs must be higher than that of 14:0. To attain these levels, 14:1(n-6)-CoA has not been tested, 14:1(n-6)-CoA is a more likely candidate for the major 14:2-6 precursor in the retina. Preliminary results from incubation of [3H]14:0-6 (Du Pont NEN) with frog retinas (n = 2) showed production of [3H]16:3-6 (3%) and [3H]14:2-6 (2%) with no detectable [3H]18:3-6 (data not shown), supporting this hypothesis.

N-terminal acylated retina proteins do not contain 14:3-6 (Johnson et al., 1994), even though n-3 polyunsaturated fatty acids are abundant in the vertebrate retina (Fliesser and Anderson, 1983). We found that metabolism of [3H]18:3-6 did not produce detectable [3H]16:3-6 or [3H]14:3-6 retroconversion products and radiolabel was not incorporated into Gtα. This result shows that the retroconversion process in the frog retina has a selectivity dependent on double bond position, as well as on chain length. The 18:3-6 did undergo extensive elongation and desaturation, following the steps toward 22:6-3, as described for liver (Sprecher, 1972), consistent with previous observations in the frog retina (Wang and Anderson, 1993).

Under the in vitro incubation conditions we used, there was an active uptake and metabolism for all the radiolabeled fatty acids, as indicated by the amount of the original starting radioactivity that was incorporated into the retina glycerolipids ([3H]14:0, 12%; [3H]18:1-9, 14%; [3H]18:2-6, 5% and [3H]18:3-6, 3%). In all incubations, the phospholipids contained most of the radiolabel, whereas only a small percentage was incorporated into triglycerides, consistent with previous studies (Wang and Anderson, 1993; Chen and Anderson, 1993b). Incorporation into glycerolipids indicates effective conversion to precursor CoA ester derivatives. Acyl-CoAs are also the substrates for myristoyltransferase, and labeling of Gtα with [3H]14:0 suggested efficient conversion to the CoA ester.

In addition to incorporation of [3H]14:0, [3H]14:1-9, and [3H]14:2-6 into Gtα, we also noted some labeling with [3H]18:1-9. We have not determined whether this represents N-terminal acylation by myristoyltransferase, which in yeast can utilize 18:1-5 – 2% as effectively as 14:0 (Rudnick et al., 1992), or trace labeling by thioesterification or some other means. Our ability to incorporate [3H]14:2-6 into Gtα is consistent with the data of Johnson et al. (1994), which shows 100% modification with 14:2-6. The incorporation of [3H]14:0 and [3H]14:1-9 into Gtα indicates that at least some Gtα in frog

**DISCUSSION**

N-terminal fatty acylation of a protein is carried out cotranslationally by the enzyme myristoyl-CoA-protein N-myristoyltransferase (Towler et al., 1988 and Schlesinger, 1993). Human and yeast myristoyltransferases have a high substrate specificity for 14:0-CoA (Kishore et al., 1991, 1993; Lu et al., 1994). Although 14:2(n-6)-CoA has not been tested, 14:1(n-9)-CoA is utilized by both myristoyltransferases at a 3-fold lower catalytic efficiency (Vmax/Km) than 14:0-CoA (Kishore et al., 1993). Despite extensive study, no evidence has been found for myristoyltransferase isozymes with drastically altered substrate specificity, and supporting this mRNA from the single copy human gene appears to be identical in all tissues studied (Duronio et al., 1992). Consequently, to successfully compete for myristoyltransferase, levels of available 14:1-9 and 14:2-6 CoAs must be higher than that of 14:0. To attain these levels, the retina would need to possess special pathways for generating 14:1-9 and 14:2-6, which are apparently absent or less active in other tissues. However, this does not mean 14:1-9 and 14:2-6 would necessarily accumulate in the retina, because their CoAs might be taken up rapidly by the myristoyltransferase during active protein synthesis. This hypothesis is consistent with the lack of detectable (<0.1%) 14:1-9 and 14:2-6 in the total lipid pool of the frog and bovine retina (Chen and Anderson, 1993a; Bartley et al., 1962).

We first tested whether 14:1-9 and 14:2-6 might be generated by de novo synthesis, because the 14:1-5 isomer can be synthesized via a Δ5-desaturase (Hamosh and Bitman, 1992; Keitzko et al., 1992), but we found that 14:0 does not serve as a desaturase substrate in the retina and only 16:0 and 18:0 are produced. We next considered that 14:1-9 and 14:2-6 might be produced during the β-oxidation (retroconversion) of long chain unsaturated fatty acids. Our experiments showed that retroconversion of 18:1-9 and 18:2-6 leads to the formation of 14:1-9 and 14:2-6, respectively, in the frog retina. Interestingly, 18:1-9 was only metabolized to 14:1-9 and 16:1-9, suggesting a restricted function for this fatty acid in the retina. Our results showed 18:2-6 has multiple roles in the retina; it was retroconverted to 14:2-6 and 16:2-6 and also underwent extensive elongation and desaturation, following the steps toward 20:4-6 as in liver (Sprecher, 1972).

The fatty acid composition of frog retinas (Chen and Anderson, 1993a) suggests that 18:1-9 and 16:1-9 are the likely precursors of 14:1-9, because they are the only n-9 fatty acids present in reasonable abundance (12 and 3% of total fatty acid, respectively). In contrast, multiple n-6 species are present, with 20:4-6 being the most abundant (7%) and 18:2-6 much less so (1%). Because rat liver peroxisomes are known to convert 20:4-6 to 14:2-6 with 18:3-6 and 16:3-6 intermediates (Luthria et al., 1995), 20:4-6 is a more likely candidate for the major 14:2-6 precursor in the retina. Preliminary results from incubation of [3H]20:4-6 (Du Pont NEN) with frog retinas (n = 2) showed production of [3H]16:3-6 (3%) and [3H]14:2-6 (2%) with no detectable [3H]18:3-6 (data not shown), supporting this hypothesis.

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retina can be modified with these fatty acids, possibly at levels too low to be detected by mass spectrometry.

Amide linkage of the \[^{14}C\]14:0, \[^{14}C\]14:1-9, and \[^{14}C\]14:2-6 to G₉ₐ, was supported by finding that the radiolabel was resistant to hydroxylamine, under conditions where 86% removal of rhodopsin’s thioester-linked fatty acid was achieved. Taken together with the mass spectrometric results of Johnson et al. (1994) and the well established specificity of myristoyltransferase in yeast and mammals, this result strongly suggests that the most likely site for attachment of these fatty acids is the α-amino group of the N-terminal glycine of frog G₉ₐ.

Because frog G₉ₐ is reported to be modified exclusively by 14:2-6, we investigated whether radiolabeled 14:2-6 would directly incorporate into the protein. We performed in vitro incubations of [1-\[^{14}C\]14:2-6 (data not shown). Analysis of the retina total lipids showed significant chain elongation of the [14C]14:2-6 to [16C]16:2 and [15C]18:2-6 (3 and 4% of [14C]14:2-6, respectively). Metabolism of nonradiolabeled 14:2-6 to 16:2 and 18:2-6 has been previously noted in rat liver (Sprecher, 1967). Methanalysis of SDS-PAGE-purified G₉ₐ (19 ± 2 μg) and G₉ₐ (13 ± 1 μg) from the [14C]14:2-6 labeled retina failed to release any detectable radiolabeled fatty acids. Because the [14C]14:2-6 used in our incubations was of very low specific activity (55–60 Ci/mol), it is possible that dilution with endogenously produced 14:2-6 precluded our detecting radioactivity in protein product. However, it is also possible that 14:2-6 supplied directly may not be readily available as a substrate for myristoyltransferase to incorporate into G₉ₐ. It is clearly converted into the necessary chemical form, CoA ester, as evidenced by chain elongation products and its incorporation into glycerolipids. However, subcellular compartmentalization may limit the access of myristoyltransferase and the nascent G₉ₐ polypeptide to 14:2-6 when it is supplied directly, while allowing free access to the 14:2-6 pool produced by retroconversion.

The fatty acid retroconversions seen in our experiments have the limited chain shortening characteristics associated with peroxisomal β-oxidation (Schulz, 1991), as shown by the lack of production of 12:1-9 and 12:2-6 or other short chain fatty acids. In support of this are electron microscopy studies showing that Müller and photoreceptor cells of frog retina contain significant peroxisome-like organelles (St. Jules et al., 1992). However, more experiments are necessary to determine where the observed β-oxidation occurs. Retina peroxisomes may prove to be more vigorous or less stringent in retroconverting these fatty acids compared with those from other tissues. Such differences may account for the absence of 14:1-9 or 14:2-6 on myristoylated liver proteins such as cytochrome b₅ reductase (Ozols et al., 1984), even though liver peroxisomes carry out fatty acid retroconversions.

Although we do not know the functional significance for N-terminal fatty acylation of photoreceptor proteins with unsaturated forms of myristate, the retroconversion pathways we have investigated could play a major role in both normal visual function and retinal disease states. Congenital defects such as adrenomyeloneuropathy (Moser et al., 1987), neonatal adrenoleukodystrophy (affe et al., 1982), infantile Refsum's disease (Poll-the et al., 1987), and Zellweger syndrome (Bowen et al., 1964) are known afflictions where peroxisomal β-oxidation is impaired. The general phenotype of these diseases is deterioration of nervous system, often involving the retina. These symptoms may arise in part from impairment of the biosynthesis of docosahexaenoic acid (22:6-3) (Martinez et al., 1994), an essential component of neuronal cells including retina photoreceptors, which requires the peroxisomal based retroconversion of 24:6-3 to 22:6-3 (Vass et al., 1991). Because N-terminal fatty acylation with 14:1-9 and 14:2-6 may be required for proper function of phototransduction proteins, impairment of 14:1-9 or 14:2-6 production could also have a devastating effect on normal visual function. It is known that many types of retinal degeneration involve lipidated phototransduction proteins, such as rhodopsin (autosomal dominant and autosomal recessive retinitis pigmentosa) (Dryja et al., 1990; Rosenfeld et al., 1992) and the β-subunit of cGMP phosphodiesterase (PDE β) (autosomal recessive retinitis pigmentosa) (McLaughlin et al., 1993). A specific example of defective protein lipidation in retinal degeneration is choroideremia, whose basis is a mutation in geranylgeranyltransferase (Seabra et al., 1993), the enzyme that isoprenylates PDE β and G proteins of the Rab family. Therefore, genes encoding proteins involved in the pathways required for heterogeneous fatty acylation of retina protein warrant further consideration as retinal degeneration candidates.

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Fig. 6. Distribution of desaturation, elongation, or retroconversion products in lipid classes derived from retina membrane pellets. Total lipids obtained from \[^{14}H\]14:0, \[^{14}H\]14:1-9, \[^{14}H\]18:2-6, and \[^{14}H\]18:3-3 radiolabeled retina membranes were resolved into phospholipids (PL), free fatty acids (FFA), and triglycerides (TG) using one-dimensional two-step TLC. Lipid classes were directly counted to determine total radioactivity. Lipid classes were then saponified, and the resulting fatty acids were converted to phenacyl esters and chromatographed on HPLC. The data are represented as percentages calculated by taking the ratio of radioactivity for the individual lipid classes or fatty acid species to the total radioactivity in the original total lipid extract or separated lipid class.

| Lipid | PL | FFA | TG |
|-------|----|-----|----|
| % Total Lipid Radioactivity | % Total Lipid Radioactivity | % Total Lipid Radioactivity |
| 14:0 | 81.3 ± 0.3 | 15.3 ± 0.3 | 2.6 ± 0.4 |
| 14:0 | 14:0 | 14:0 |
| 7 | 9 | 4 |
| 3 | 2 | 2 |
| % Total Lipid Radioactivity | % Total Lipid Radioactivity | % Total Lipid Radioactivity |
| 18:1-9 | 18:1-9 | 18:1-9 |
| 77.5 ± 0.2 | 18.8 ± 0.5 | 3.6 ± 0.3 |
| 14:1 | 14:1 |
| 2 | 1 | 0.3 |
| 4 | 3 | 3 |
| 71 | 82 | 88 |
| % Total Lipid Radioactivity | % Total Lipid Radioactivity | % Total Lipid Radioactivity |
| 18:3-3 | 18:3-3 | 18:3-3 |
| 82.2 ± 4.5 | 12.2 ± 3.3 | 4.9 ± 1.4 |
| 12 | 8 | 11 |
| 8 | 5 | 7 |
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James C. Jr. DeMar, Theodore G. Wensel and Robert E. Anderson

J. Biol. Chem. 1996, 271:5007-5016.
doi: 10.1074/jbc.271.9.5007

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