9-19-1997

An Animal Cell Mutant With a Deficiency in Acyl/Alkyl-Dihydroxyacetone- Phosphate Reductase Activity. Effects on the Biosynthesis of Ether-Linked and Diacyl Glycerolipids

Paul F. James
Boston University

Andrew C. Lake
Boston University

Amiya K. Hajra
University of Michigan, Ann Arbor

Leslie K. Larkins
University of Michigan, Ann Arbor

Mitchell Robinson
Quillen-Dishner College of Medicine

See next page for additional authors

Citation Information

James, Paul F.; Lake, Andrew C.; Hajra, Amiya K.; Larkins, Leslie K.; Robinson, Mitchell; Buchanan, F. Gregory; and Zoeller, Raphael A.. 1997. An Animal Cell Mutant With a Deficiency in Acyl/Alkyl-Dihydroxyacetone- Phosphate Reductase Activity. Effects on the Biosynthesis of Ether-Linked and Diacyl Glycerolipids. *Journal of Biological Chemistry*. Vol.272(38). 23540-23546. https://doi.org/10.1074/jbc.272.38.23540 PMID: 9295290 ISSN: 0021-9258

An Animal Cell Mutant With a Deficiency in Acyl/Alkyl-Dihydroxyacetone-Phosphate Reductase Activity. Effects on the Biosynthesis of Ether-Linked and Diacyl Glycerolipids

Creator(s)

Paul F. James, Andrew C. Lake, Amiya K. Hajra, Leslie K. Larkins, Mitchell Robinson, F. Gregory Buchanan, and Raphael A. Zoeller

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 38, Issue of September 19, pp. 23540–23546, 1997
Printed in U.S.A.

An Animal Cell Mutant with a Deficiency in Acyl/Alkyl-dihydroxyacetone-phosphate Reductase Activity

EFFECTS ON THE BIOSYNTHESIS OF ETHER-LINKED AND DIACYL GLYCEROLIPIDS*

(Received for publication, March 21, 1997, and in revised form, June 25, 1997)

Paul F. James‡§, Andrew C. Lake‡, Amiya K. Hajra¶, Leslie K. Larkins¶, Mitchell Robinson‖, F. Gregory Buchanan‖, and Raphael A. Zoeller‡**

From the ‡Department of Biophysics, Boston University School of Medicine, Boston, Massachusetts 02118, the ¶Department of Biological Chemistry and Mental Health Institute, University of Michigan, Ann Arbor, Michigan 48109, and the ‖Department of Biochemistry, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614

In the accompanying paper (James, P. F., and Zoeller, R. A. (1997) *J. Biol. Chem.* 272, 23532–23539), we reported the isolation of a series of mutants from the fibroblast-like cell line, CHO-K1, that are deficient in the incorporation of the long chain fatty alcohol, hexadecanol, into complex lipids. All but one of these mutants, FAA.K1B, were deficient in long-chain-fatty alcohol oxidase (FAO) activity. We have further characterized this FAO$^+$ isolate. FAA.K1B cells displayed a 40% decrease in [9,10-^{3}H]hexadecanol uptake when compared with the parent strain. Although incorporation of hexadecanol into the phospholipid fraction was decreased by 52%, the cells accumulated label in alkylglycerol (20-fold over wild type). The increase in 1-alkylglycerol labeling corresponded to a 4-fold increase in alkylglycerol mass. Short term labeling with $^{32}P_i$ showed a 45–50% decrease in overall phospholipid biosynthesis in FAA.K1B. Both diacyl- and ether-linked species were affected, suggesting a general defect in phospholipid biosynthesis. Mutant cells were able to partially compensate for the decreased biosynthesis by decreasing the turnover of the phospholipid pools. The primary lesion in FAA.K1B was identified as a 95% reduction in acyl/alkyl-dihydroxyacetone-phosphate reductase activity. Whole cell homogenates from FAA.K1B were unable to reduce either acyl-dihydroxyacetone phosphate (DHAP) or alkyl-DHAP, supporting the notion that the reduction of these two compounds is catalyzed by a single enzyme. These data suggest that the biosynthesis of diacyl phospholipids, in Chinese hamster ovary cells, begins with the acylation of dihydroxyacetone phosphate as well as glycero-3-phosphate and that the "DHAP pathway" contributes significantly to diacyl glycerolipid biosynthesis. Also, the severe reduction in acyl/alkyl-DHAP reductase activity in FAA.K1B resulted in only a moderate decrease in ether lipid biosynthesis. These latter data together with the observed increase in alkylglycerol levels support the existence of a shunt pathway that is able to partially bypass the enzymatic lesion.

The formation of phosphatidic acid is required for the formation of diacyl glycerolipids in both bacterial and animal cells. In bacterial systems, this is initiated with the reduction of dihydroxyacetone phosphate (DHAP),[1] a product of glycolysis. The resulting *sn*-glycero-3-phosphate is acylated sequentially at the *sn*-1 and *sn*-2 positions to form phosphatidic acid. In animal cells, dihydroxyacetone phosphate (DHAP) can be acylated directly. In this "DHAP pathway," the ketone at the *sn*-2 position of the resulting *sn*-1-acyl-DHAP must be reduced, by acyl/alkyl-DHAP reductase (1), prior to further acylation. Purified acyl/alkyl-DHAP reductase also catalyzes the reduction of the ether-linked *sn*-1-alkyl-DHAP (2), a reaction that is required in the synthesis of ether-linked glycerolipids, such as plasmenylethanolamine and glyceryl ether diesters (1). While the acylation of DHAP is accepted as the first step in ether-linked glycerolipids, the importance of the DHAP pathway and acyl/alkyl-DHAP reductase in the synthesis of diacyl glycerolipids is in question. It has been estimated that this route is a significant contributor to diacyl glycerolipid synthesis (3, 4), and other studies suggest that this pathway plays a minor role (5–7).

Acyl/alkyl-DHAP reductase is a membrane-bound enzyme that has been localized to the cytosolic face of both the peroxisomal (8) and microsomal membranes (9). Interestingly, in both human (10, 11) and rodent (12, 13) cells that do not assemble peroxisome properly, other activities associated with the peroxisomal membrane, such as DHAP acyltransferase and alkyl-DHAP synthase, are severely decreased, but acyl/alkyl-DHAP reductase activity remains unaffected. Although it has been purified to homogeneity (2), the amino acid sequence for this enzyme has not been determined, the gene has not been isolated, and there is no information concerning how this enzyme is targeted.

We have isolated a mutant, FAA.K1B, that displays a general defect in phospholipid biosynthesis and an increase in 1-alkylglycerol levels. Further characterization showed this cell line to be defective in acyl/alkyl-DHAP reductase activity. The observation that these mutants were reduced in the biosynthesis of diacyl phospholipids suggests that the DHAP pathway plays a significant role in the production of these lipids in

* This research was supported by National Institutes of Health Grants GM 50571 (to R. A. Z.), NS 15747 (to A. K. H.), and CA 59981 (to M. R.) and a Boston University Graduate Student Research award (to P. F. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, P.O. Box 670524, Cincinnati, OH 45267-0524.

** To whom correspondence should be addressed: Dept. of Biophysics, Center for Advanced Biomedical Research, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118. Tel.: 617-638-4010; Fax: 617-638-4041.

[1] The abbreviations used are: DHAP, dihydroxyacetone-phosphate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; AG, *sn*-1-alkylglycerol.

CHO cells. These cells will be useful in investigating the interrelationship between diacyl and ether glycerolipid biosynthesis. This cell line may serve as a tool to isolate a gene that is responsible for acyl/alkyl-DHAP activity and to examine intracellular targeting of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—[1-^{3}H]Ethanolamine (28.8 Ci/mmol) was purchased from Amersham Corp. [*methyl*-^{3}H]Thymidine (84 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). [9,10-^{3}H]Hexadecanoic acid (60 Ci/mmol), $^{32}P_i$ (9000 Ci/mmol), [γ-^{32}P]ATP (30 Ci/mmol), L-[^{35}S]methionine (900 Ci/mmol) and EN^{3}HANCE spray were purchased from NEN Life Science Products. [9,10-^{3}H]Hexadecanol was synthesized from [9,10-^{3}H]hexadecanoic acid (14, 15). [^{32}P]DHAP and [^{32}P]*sn*-glycero-3-phosphate were synthesized using glycerokinase and [γ-^{32}P]ATP by the method of Schlossman and Bell (16) or by the method of Hajra and Burke (17). *B*-[4-^{3}H]NADPH was prepared as described by Hajra *et al*. (18). Acyl-DHAP and alkyl-DHAP (hexadecyl-DHAP) were prepared as described by Hajra *et al*. (19). All other biochemicals were purchased from Sigma unless otherwise specified.

Cells and Culture Conditions—CHO-K1 (wild-type) cells were obtained from the American Type Culture Collection (Rockville, MD). FAA.K1B is a variant of CHO-K1 that was isolated from a population of cells that were defective in the ability to take up long chain fatty alcohol (20). All cell lines were routinely cultured in Ham's F12 nutrient mix (BioWhittaker) containing 10% fetal bovine serum (BioWhittaker), glutamine (1 mM), penicillin G (100 units/ml), and streptomycin (75 units/ml). This growth medium is designated "F12c" throughout the text. Cells were cultured at 37 °C unless otherwise specified.

Accumulation and Distribution of [9,10-^{3}H]Hexadecanol—Cells were plated into sterile, 20-ml glass scintillation vials, at 2×10^5 cells/vial, in 1 ml of F12c and allowed to attach overnight at 37 °C. The following day, 0.5 ml of F12c containing 15 μM [9,10-^{3}H]hexadecanol (2×10^6 cpm/vial) was added to each vial (final hexadecanol concentration of 5 μM), and the cells were incubated at 37 °C for 1.5 or 3 h. Medium was removed, the cells were washed 2 times with 3 ml of F12c followed by one wash with 3 ml of PBS. Lipids were extracted from the cell monolayers by the method of Bligh and Dyer (21). In brief, 3.8 ml of chloroform:methanol:PBS (1:2:0.8) containing 300 μg of carrier lipid (total lipid extract from bovine heart) was added to the scintillation vial containing the washed monolayer. This single phase mixture was transferred to a test tube, and a two-phase system was formed by the addition 1 ml of chloroform and 1 ml of PBS followed by vortexing. After separation of the phases by centrifugation, the lower (organic) phase was recovered. The upper phase was washed once with 2 ml of pre-equilibrated lower phase; the lower phases were combined and dried under a stream of nitrogen, and the final samples were resuspended in 1 ml of chloroform. An aliquot of the extracted lipids was transferred to a scintillation vial, the chloroform was evaporated, the lipids were re-solubilized in methanol and quantitated by liquid scintillation spectrometry, following the addition of scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA). Duplicate, unlabeled vials were used for protein determination. Protein was determined by the method of Lowry *et al.* (22).

To determine which lipid species were labeled, aliquots of each sample were spotted on silica gel 60 thin layer chromatography plates (Merck) and developed in one of several solvent systems. Phospholipids were separated using a two-dimensional thin layer chromatography (TLC) system (23) using chloroform:methanol:acetic acid:H_2O (25:15:3:1.5) in the first dimension, followed by development in the second dimension using chloroform:methanol, 88% formic acid (65:25:10). The plates were sprayed with 10 mM $HgCl_2$ in acetic acid and allowed to dry for 30 min in a fume hood between dimensions. This cleaved the vinyl ether within plasmenylethanolamine (24), allowing the resulting *sn*-1-lysophosphatidylethanolamine and fatty aldehyde to be separated from the unaffected diacyl phosphatidylethanolamine in the second dimension. Neutral lipids were separated by development in a single dimension in *n*-hexane:diethyl ether:acetic acid (70:30:1). Alkylglycerols were resolved from all other lipid species by development in *n*-hexane:diethyl ether:acetic acid (20:80:1), followed by a second development in the same dimension using isopropyl ether:acetic acid (96:4). Following separation by TLC, the plates were sprayed with EN^{3}HANCE and exposed to x-ray film at −70 °C. Bands of interest were scraped into scintillation vials containing 1 ml of methanol, and the amount of radioactivity associated with each lipid species was determined by liquid scintillation spectrometry after the addition of scintillation fluid. Co-migration with authentic standards was used to identify lipid species. The putative 1-alkylglycerol co-migrated with authentic *sn*-1-hexadecylglycerol in all of the solvent systems described above.

Phospholipid Biosynthesis—Cells were plated into sterile glass scintillation vials at 2.5×10^5 cells/vial and allowed to attach overnight at 37 °C. The cells were then incubated for 2.5 and 5 h at 37 °C in growth medium containing $^{32}P_i$ at 60 μCi/ml. Labeling medium was removed, and the cellular lipids were recovered from the monolayers as described above for fatty alcohol uptake studies. An aliquot of the sample was used for determination of total phospholipid biosynthesis. The remaining labeled phospholipids were separated using the two-dimensional TLC system described above. The bands of interest were localized by autoradiography; labeled lipids were scraped into scintillation vials containing 1 ml of methanol, and radioactivity was quantitated by liquid scintillation spectrometry as described above.

Phospholipid Turnover—Cells were plated into sterile glass scintillation vials at 2.5×10^5 cells/vial and incubated for 12 h at 37 °C in growth medium containing $^{32}P_i$ at 10 μCi/ml. Labeling medium was removed and replaced with unlabeled growth medium. After 0, 2.5, 5, 10, and 26 h at 37 °C, medium was removed, and the cellular lipids were recovered from the cell monolayers as described above for fatty alcohol uptake studies. The dried lipids were resuspended in 1 ml of chloroform, and an aliquot was transferred to a scintillation vial. After the chloroform was allowed to evaporate, 1 ml of methanol was added, followed by 8 ml of scintillation fluid, and the radioactivity was determined using liquid scintillation spectrometry.

Alternatively, a longer labeling period was used, to steady-state label the phospholipid pools, prior to chase. The cells were labeled using $^{32}P_i$ (5 μCi/ml) for 72 h in a tissue culture flask. The cells were harvested and plated into scintillation vials at 5×10^4 cells/vial. After allowing the cells to attach for 4 h, the amount of label associated with cellular phospholipids was determined, as above, at 24-h intervals.

Phospholipid Analysis—Cells were plated at 10^6 cells/100-mm diameter tissue culture dish in 15 ml of growth medium and allowed to grow for 48 h at 37 °C. At this point, cultures were still subconfluent. For each dish, medium was removed, the cells were washed once with 7 ml of PBS, and the cells were detached from the dishes, using a rubber policeman, into 5 ml of PBS. For each sample, cells from four dishes were combined and pelleted by centrifugation at $600 \times g$ for 7 min. The final pellet was resuspended in 1.0 ml of PBS. An aliquot of this suspension was used for protein determination, and 0.8 ml were transferred to 3 ml of chloroform:methanol (1:2). The lipids were extracted using a neutral Bligh and Dyer extraction (21) and separated using the two-dimensional TLC system described above for fatty alcohol uptake experiments using silica gel G plates (Analtech). After separation, the plates were sprayed with 50% sulfuric acid and charred on a heating plate. Individual phospholipid species were scraped into test tubes and the phosphorous was quantitated by the method of Rouser *et al*. (25).

Cellular Levels of Alkylglycerol and Monoacylglycerol Species—Cells were grown to near confluence in tissue culture dishes. Medium was removed, and the cell monolayers were washed with PBS and harvested with trypsin, and the cell number was determined using a hemocytometer. The cells were centrifuged at $600 \times g$ for 7 min and resuspended in PBS. The cellular lipids were extracted using the method of Bligh and Dyer (21). The lipids were spotted on silica gel H TLC plates (Analtec) and developed in *n*-hexane:ethyl ether:acetic acid (40:60:1). The band corresponding to the combined alkylglycerols and monoacylglycerols was scraped and extracted from the silicic acid, using a Bligh and Dyer system, after the addition of 12:0-monoacylglycerol and 12:0-alkylglycerol as internal standards. The lipids were dried under nitrogen, and the alkylglycerols and monoacylglycerols were converted to their isopropylidine derivatives (26). The individual species were separated by gas-liquid chromatography (27, 28) and quantitated by comparison of the peak areas to those of the internal standard.

Enzymatic Assays—Frozen whole cell homogenates were used in all assays. Cells were grown to near confluence in 100-mm diameter tissue culture dishes in F12c at 37 °C. For each dish, medium was removed, the cells were washed twice with ice-cold phosphate-buffered saline (PBS), and scraped into 3 ml of PBS. The cells were pelleted by centrifugation at $600 \times g$ for 7 min, washed once with 5 ml of PBS, and resuspended in 1 ml of homogenization buffer (25 mM Tris-HCl, pH 8.0, 0.25 M sucrose). The cell suspension was homogenized with 15 up and down strokes of a motorized glass-Teflon homogenizer and frozen at −70 °C. Protein content of each homogenate was determined by the method of Lowry (22). Homogenates were thawed on ice and sonicated for 30 s using a sonic water bath (Branson Utlasonics Corporation, Danbury, CT; model 2210) prior to assays.

DHAP acyltransferase and glycero-3-phosphate acyltransferase activities were assayed as described previously by Jones and Hajra (29).

Alkyl-DHAP synthase activity was assayed by measuring the incorporation of [1-^{14}C]hexadecanol into alkyl-DHAP in the presence of 1-acyl-DHAP, as described by Davis and Hajra (14). Acyl/alkyl-DHAP reductase was monitored by measuring the incorporation of label from *B*-[4-^{3}H]NADPH into chloroform-soluble counts in the presence of 1-*O*-hexadecyl-DHAP (30) or 1-palmitoyl-DHAP (2).

Ethanolamine Labeling of Phospholipids—Cells were plated into sterile glass scintillation vials at 3×10^5 cells/vial and allowed to attach overnight at 37 °C. The cells were then incubated for 2 or 4 h at 37 °C in growth medium containing [1-^{3}H]ethanolamine (approximately 1 μCi/ml). Labeling medium was removed, and the cellular lipids were recovered from the monolayers, as described above, for fatty alcohol uptake studies. The labeled phospholipids were separated using a double-development, single-dimension TLC system (31). This separated the labeled phospholipids into three bands as follows: band 1, plasmenylethanolamine; band 2, phosphatidylethanolamine; and band 3, and minor, unidentified lipid that traveled near the solvent front. The bands of interest were localized by fluorography and quantitated by liquid scintillation spectrometry as described above. The majority (>95%) of the label was found in bands 1 and 2. When the cells were supplemented with 1-hexadecylglycerol (alkylglycerol; AG), this compound was added to the labeling medium from a 20 mM stock (in ethanol). The medium was sonicated for 5 min in a sonicating bath just prior to addition of the labeling medium.

DNA and Protein Synthesis—The rate of DNA and protein synthesis was assayed by measuring the rate of incorporation of [methyl-^{3}H]thymidine and [^{35}S]methionine into trichloroacetic acid-insoluble material (32). Cells were plated into 24-well plates at 5×10^4 cells/well and allowed to attach overnight at 37 °C. Either [^{35}S]methionine (6 μCi) or [*methyl*-^{3}H]thymidine (2.5 μCi) were added to the wells, and the cells were incubated at 37 °C. After 3 h, medium was removed, and 1 ml of ice-cold 10% trichloroacetic acid was added. The cell monolayer was washed three times with ice-cold trichloroacetic acid followed by four washes with ice-cold ethanol:ether (3:1). After drying, 0.5 ml of 0.5 N NaOH was added to solubilize the cell material at 37 °C for 2 h. Aliquots were used for liquid scintillation counting.

RESULTS

Uptake and Distribution of [9,10-^{3}H]Hexadecanol into Complex Lipids—FAA.K1B was isolated from a mutagenized population of CHO-K1 that had survived a tritium suicide selection protocol designed to select cells that were less able to take up [9,10-^{3}H]hexadecanol into stable pools of lipid (20). All, except FAA.K1B, were characterized as having a defect in fatty-alcohol oxidase activity.

FAA.K1B was significantly reduced in its ability to take up hexadecanol from the medium (Fig. 1), displaying a 40% decrease when compared with CHO-K1. The metabolic fate of hexadecanol that was taken up by FAA.K1B was examined. Following 3 h of labeling the cells with [9,10-^{3}H]hexadecanol, the lipids were extracted, and the individual lipid species were separated using thin layer chromatography (Fig. 2). Quantitation (Table I) showed that labeling of every phospholipid class was reduced in FAA.K1B, particularly in the choline phospholipids (phosphatidylcholine). Incorporation of hexadecanol into the *sn*-1 position of the ether-linked phospholipid, plasmenylethanolamine (observed as a fatty aldehyde upon $HgCl_2$ treatment), was also greatly reduced. Within the neutral lipids, cholesterol ester and triglyceride labeling was similar in FAA.K1B and wild-type cells, but the incorporation of label into glyceryl ether diesters was severely reduced in these mutants.

The most striking anomaly in the labeling pattern of FAA.K1B was the appearance of a labeled lipid species that traveled with the neutral lipids (Fig. 2). The labeling in this lipid species was increased in FAA.K1B by 21–22-fold over wild-type cells and represented approximately 40% of the label incorporated into complex lipids in FAA.K1B (Table I). Based on several criteria, we identified this lipid species as alkylglycerol. These criteria included the following: 1) co-migration of the labeled compound with authentic *sn*-1-hexadecylglycerol on TLC using four solvent systems (see "Experimental Procedures"); 2) resistance to mild-base hydrolysis; and 3) labeling

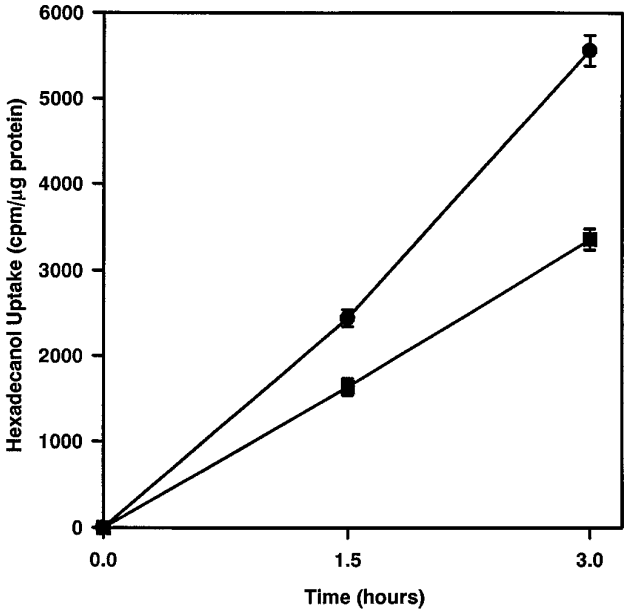

FIG. 1. **Uptake of hexadecanol into CHO-K1 and FAA.K1B cells.** Cells were incubated in serum-containing medium containing 5 μM [9,10-^{3}H]hexadecanol at 37 °C. Following incubation, medium was removed, and the cells were washed and the radioactive lipids extracted as described under "Experimental Procedures." Each value represents the average of three determinations ± S.D. ●, CHO-K1; ■, FAA.K1B.

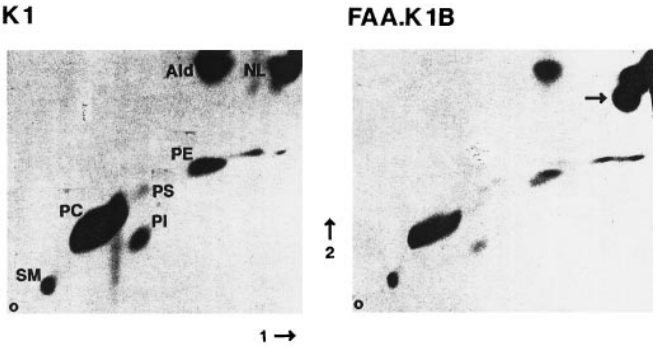

FIG. 2. **Distribution of label from [9,10-^{3}H]hexadecanol into complex lipids.** Cells were incubated for 3 h in 5 μM [9,10-^{3}H]hexadecanol as in Fig. 1. After extraction, lipids were separated into individual lipid species by two-dimensional thin layer chromatography as described under "Experimental Procedures." The area containing lipids was treated with $HgCl_2$ between dimensions to cleave the vinyl ether double bond of plasmenylethanolamine. This resulted in a lysophospholipid and a long chain fatty aldehyde leaving the diacyl phospholipid (phosphatidylethanolamine) unaffected. The abbreviations used are: *O*, origin; *SM*, sphingomyelin; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PE*, phosphatidylethanolamine; *NL*, neutral lipids; *Ald*, fatty aldehyde (generated from the *sn*-1 position of plasmenylethanolamine after development in the first dimension). *Arrow* within the *right panel* indicates the accumulation of label in alkylglycerols in FAA.K1B. *Numbered arrows* on the perimeter indicate the directions of the first and second developments.

with hexadecanol but not hexadecanoic acid or $^{32}P_i$ (not shown). Isopropylidine derivatization of the neutral lipid fraction followed by analysis by gas-liquid chromatography showed a 4-fold increase in alkylglycerol levels in FAA.K1B over wild-type cells (Table II). There was only a slight increase in the level of monoacylglycerols.

Phospholipid Biosynthesis, Turnover, and Composition—Short term labeling of cells, using $^{32}P_i$, showed a 45–50% decrease in the rate of phospholipid biosynthesis in FAA.K1B compared with wild-type cells (Fig. 3). Examination of the individual phospholipid species showed that the labeling of all phospholipid species, except phosphatidylethanolamine, was

TABLE I

Distribution of label from [9,10-^{3}H]hexadecanol into individual phospholipid and neutral lipid species

Cells were labeled with 5 μM [9,10-^{3}H]hexadecanol for 3 h at 37 °C as described under "Experimental Procedures." After extraction of the lipids (21), the individual lipid species were isolated using three different systems on thin layer chromatography. Phospholipids were separated using two-dimensional thin layer chromatography as shown in Fig. 2. All neutral lipids, except alkylglycerol (AG), were separated using *n*-hexane:diethyl ether:acetic acid (80:20:1). To isolate AG a fraction of the extracted lipids were developed first, using *n*-hexane:diethyl ether:acetic acid (20:80:1) followed by a second development in isopropyl ether:acetic acid (96:4). Plasmenylethanolamine (Plasmenyleth.) aldehyde is the result of $HgCl_2$ cleavage of the vinyl ether linkage between TLC runs during the two-dimensional TLC (see legend to Fig. 2). Abbreviations are: SM, sphingomelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; AG, *sn*-1-alkylglycerols; TAG, triacylglycerol; GEDE, glycerol ether diester; CE, cholesterol ester. All values represent the average ± S.D. of three samples.

	Radioctivity										
	Phospholipids						Neutral lipids				
	SM	PC	PI	PS	PE	Plasmenyleth. aldehyde	AG	TAG	GEDE	CE	Fatty alcohol
	cpm / μg protein										
CHO-K1	77 ± 8	1,289 ± 150	66 ± 8	18 ± 1	197 ± 16	432 ± 46	24 ± 1	184 ± 2	387 ± 13	62 ± 8	787 ± 60
FAA.K1B	33 ± 2	215 ± 27	21 ± 2	15 ± 3	73 ± 22	127 ± 16	523 ± 12	161 ± 8	125 ± 3	56 ± 10	850 ± 46

TABLE II

Alkylglycerol and monoacylglycerol levels in CHO-K1 and FAA.K1B

Species	Alkylglycerol levels		Monoacylglycerol levels	
	CHO-K1	FAA.K1B	CHO-K1	FAA.K1B
	pmol / 10^6 cells		*pmol / 10^6 cells*	
14:0			17.1 ± 5.2	28.6 ± 10.0
16:0	68.4 ± 11.7	353.7 ± 110.7	102.5 ± 16.5	120.7 ± 45.2
18:0	29.0 ± 5.5	33.0 ± 16.5	232.1 ± 101.5	343.7 ± 98.6
18:1	18.7 ± 9.1	50.1 ± 4.9	5.4 ± 8.2	10.8 ± 12.4
Total	116.1 ± 17.6	436.8 ± 138.0	357.1 ± 107.7	503.8 ± 138.0

Cellular levels of the individual alkylglycerol and monoacylglycerol species were determined as described under "Experimental Procedures." All values represent the average ± S.D. of four separate determinations.

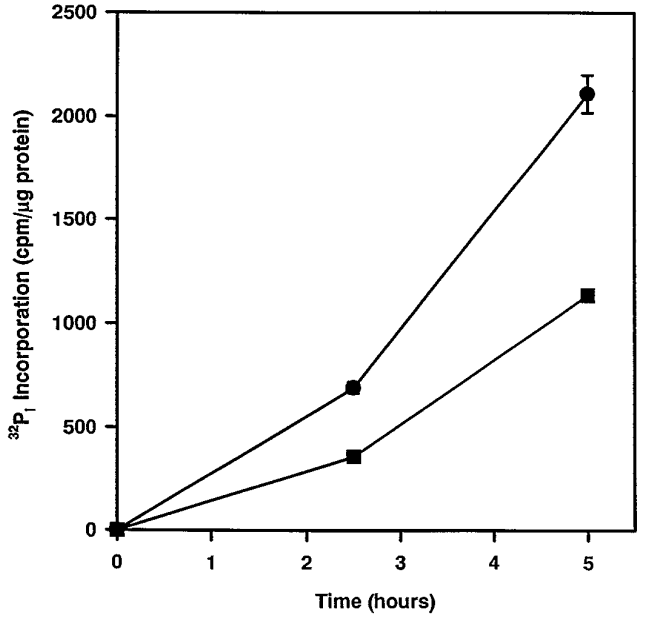

FIG. 3. **Phospholipid biosynthesis in CHO-K1 and FAA.K1B.** Cells were labeled with $^{32}P_i$ at 37 °C followed by extraction of the lipids as described under "Experimental Procedures." An aliquot of the chloroform-soluble material was used for scintillation spectrometry. All values represent the average ± S.D. of three separate samples. ●, CHO-K1; ■, FAA.K1B.

reduced (Table III). Although phospholipid biosynthesis appeared to be impaired in FAA.K1B, the ratio of phospholipid to protein was only slightly reduced (Table IV). Analysis of the phospholipid composition revealed significant, although minor, decreases in phosphatidylcholine, plasmenylethanolamine, and phosphatidylinositol and an increase in the relative amounts of sphingomyelin and phosphatidylethanolamine.

The turnover of phospholipid in FAA.K1B was also examined. When cells were labeled for only 12 h prior to chase, there was no loss of label from the newly formed phospholipids over the first 10 h (Fig. 4*A*). There was no significant difference between wild-type and mutant cells with respect to loss of label throughout the course of the 26 h following labeling. When cells were labeled for many generations to steady-state label the phospholipid pools, we observed a significantly slower turnover of phospholipid in the mutant cells (Fig. 4*B*).

Identification of the Enzymatic Lesion in FAA.K1B, as a Deficiency in Acyl/Alkyl-dihydroxyacetone-phosphate Reductase—The $^{32}P_i$ labeling patterns observed in FAA.K1B (Table III) suggested that the synthesis of both diacyl phospholipids and ether-linked phospholipids was affected in this cell line. The biosynthetic pathways for these two types of phospholipid share certain steps and they also contain steps that are unique to each. The first two steps in ether lipid biosynthesis are catalyzed by the peroxisomal enzymes, peroxisomal dihydroxyacetone-phosphate acyltransferase and alkyl-DHAP synthase (33). These two activities were normal in whole cell homogenates from FAA.K1B (Table V). DHAP acyltransferase was normal whether it was assayed at pH 5.5 or pH 7.4. The acylation of glycero-3-phosphate, by glycero-3-phosphate acyltransferase, was also normal in FAA.K1B homogenates. The third step in the pathway is catalyzed by alkyl/acyl-DHAP reductase, which reduces the ketone group at the *sn*-2 position of the backbone. This activity was reduced to 5% of wild-type values in whole cell homogenates of FAA.K1B. This reduction was observed using either acyl-DHAP or alkyl-DHAP as a substrate.

To examine steps downstream of the reductase, we bypassed the reductase lesion with supplementation of the medium with *sn*-1-hexadecylglycerol (alkylglycerol; AG). This compound readily enters cells and is phosphorylated to enter the ether lipid pathway as 1-alkyl-2-lyso-*sn*-glycero-3-phosphate, the product of the reductase. Assuming steps downstream of the reductase are intact, AG supplementation should result in bypass of the reductase lesion and restoration of ether lipid biosynthesis. AG supplementation has been used previously (34) to identify lesions downstream in the pathway for plasmenylethanolamine biosynthesis in other mutants. As shown in Fig. 5, the labeling of plasmenylethanolamine was reduced by approximately 50% in FAA.K1B, whereas the labeling of phosphatidylethanolamine was comparable to wild type. These results agree well with the labeling pattern observed when using $^{32}P_i$ (Table III). The addition of AG to the medium restored the labeling of plasmenylethanolamine to near wild-type values, suggesting that the steps downstream of acyl/alkyl-DHAP re-

TABLE III
Short term $^{32}P_i$ labeling of individual phospholipid species

Cells were labeled for 5 h with $^{32}P_i$ at 37 °C as described under "Experimental Procedures." After extraction of the lipids (21), the individual lipid species were isolated using two-dimensional thin layer chromatography. Abbreviations are: SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; pPE, plasmenylethanolamine. All values represent the average ± S.D. of three samples. The values in parentheses represent the percent of the value for CHO-K1.

	Radioactivity					
	SM	PC	PI	PS	PE	pPE
			cpm/μg protein			
CHO-K1	23 ± 5	1,016 ± 107	186 ± 36	14 ± 2	184 ± 14	134 ± 5
FAA.K1B	8 ± 2 (35)	440 ± 59 (43)	151 ± 2 (81)	9 ± 3 (64)	172 ± 17 (93)	59 ± 5 (44)

TABLE IV
Phospholipid composition of CHO-K1 and FAA.K1B

Phospholipids were extracted and the individual lipid species were isolated by two-dimensional TLC as described under "Experimental Procedures." Phospholipid mass was determined using the method of Rouser *et al.* (25). All values represent the average ± S.D. of three separate samples. For abbreviations, see legend to Table III.

	Percent of total phospholipid							PL content[a]
	SM	PC	PI	PS	PE	pPE	Other	
								μg P/mg protein
CHO-K1	9.6 ± 1.1	54.9 ± 2.4	7.3 ± 0.9	4.6 ± 1.1	12.3 ± 2.4	8.2 ± 0.4	3.1 ± 0.9	3.81 ± 0.15
FAA.K1B	14.4 ± 2.6	49.4 ± 5.1	5.8 ± 0.5	5.4 ± 2.0	16.7 ± 2.1	6.0 ± 0.8	2.4 ± 1.7	3.37 ± 0.47

[a] Values are presented as μg of phosphorous (P)/mg of cellular protein.

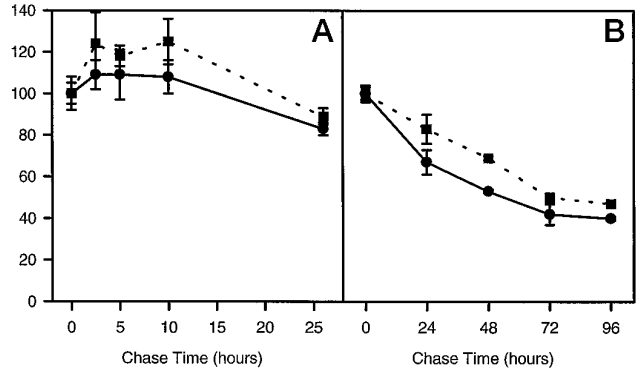

FIG. 4. **Phospholipid turnover.** The cellular phospholipids were labeled for 12 h (*A*) or 72 h (*B*) with $^{32}P_i$. Medium was removed and replaced with unlabeled growth medium. The lipids were extracted at the indicated times following fluid change, and the radioactivity associated with the lipid (chloroform-soluble counts) was determined as described under "Experimental Procedures." The data are expressed as the percent of lipid-associated radioactivity found at time = 0. All values represent the average ± S.D. of three samples. ●, CHO-K1; ■, FAA.K1B.

ductase were functioning. Interestingly, the labeling of phosphatidylethanolamine was reduced in AG-treated cells.

The biosynthesis of the diacylated phospholipids, if DHAP is used during the initial acylation step, requires this reductase activity as well. Therefore, the reduction of 1-acyl-DHAP and the steps downstream are catalyzed by the same set of enzymes as those used in ether lipid biosynthesis. Again, only the reductase step was deficient in FAA.K1B. In summary, the only lesion that could be identified in the biosynthesis of ether-linked or diacylated phospholipids was a reduction the acyl/alkyl-DHAP reductase.

DISCUSSION

FAA.K1B is a novel mutant that displayed a severe defect in acyl/alkyl-DHAP reductase. This activity catalyzes the third step in biosynthesis of ether-linked glycerolipids (33). The loss of this activity resulted in a decrease in the rate of biosynthesis of plasmenylethanolamine, although not to the extent that we would have expected (56% decrease) given a 95% decrease in acyl/alkyl-DHAP reductase activity. Lesions in peroxisomal DHAP acyltransferase activity (34) and alkyl-DHAP synthase (12), which catalyze the first and second steps, respectively, in ether lipid biosynthesis, resulted in much more dramatic effects on ether lipid biosynthesis. The observed accumulation of alkylglycerol in FAA.K1B suggests that these cells were able to partially bypass the reductase lesion through a salvage pathway. 1-Alkyl-DHAP, not reduced by the acyl/alkyl-DHAP reductase, would be dephosphorylated, followed by reduction of the resulting 1-alkyldihydroxyacetone, by a separate reductase, to form 1-alkylglycerol. This could then be phosphorylated by a kinase to re-enter the biosynthetic pathway downstream of the acyl/alkyl-DHAP reductase as 1-alkyl-*sn*-glycero-3-phosphate. All of these activities have been demonstrated in animal cells (33). This salvage pathway, by generating alkylglycerols, could partially bypass the reductase lesion. The addition of high levels of exogenous alkylglycerol to the cells (Fig. 5) was able to more completely bypass the lesion.

Acyl/alkyl-DHAP reductase can also be used during the synthesis of diacyl glycerolipids (33). The biosynthesis of glycerolipids begins by the acylation of either glycero-3-phosphate or dihydroxyacetone phosphate at the *sn*-1 carbon of the backbone. If glycero-3-phosphate is the initial acyl acceptor, the *sn*-2 carbon is acylated next, to form phosphatidic acid. If DHAP is acylated (the DHAP pathway) the ketone at the *sn*-2 carbon must be reduced by the acyl/alkyl-DHAP reductase, prior to acylation. Although the role of the reductase in ether lipid biosynthesis is known, there has been some question concerning the importance of the reductase and the DHAP pathway for diacyl glycerolipid biosynthesis (3–7). The $^{32}P_i$ labeling data (Table III) suggested that, for CHO cells, this pathway is significant. Overall, the rate of phospholipid biosynthesis was reduced by 45%. A decrease in ether lipid biosynthesis, alone, could not account for this decrease. In other ether lipid$^-$ mutants (12, 13), the decrease in ether lipid biosynthesis had no effect on the biosynthetic rates of the head group classes. In fact, the biosynthesis of the diacylated species was increased to compensate for the loss of the ether-linked species.

Although the synthesis of phosphatidylcholine was dramatically reduced in FAA.K1B, there was little effect on the biosynthesis of phosphatidylethanolamine (Table III). It is well

TABLE V

Activities of enzymes involved in the early steps in glycerolipid biosynthesis

Whole cell homogenates were prepared and assays were performed as described under "Experimental Procedures." Unless otherwise noted, all values represent the average ± S.D. of three homogenates.

Strain	Specific activity in whole cell homogenates					
	DHAP acyltransferase		G3P Acyltransferase	Alkyl-DHAP synthase	Acyl/Alkyl-DHAP reductase	
	pH 5.5	pH 7.4			Alkyl-DHAP[a]	Acyl-DHAP[b,c]
	nmol/min/mg protein					
CHO-K1	0.66 ± 0.06	1.44 ± 0.17	2.98 ± 0.17	0.112 ± 0.004	0.57 ± 0.06	0.65
FAA.K1B	0.59 ± 0.05	1.20 ± 0.11	2.92 ± 0.12	0.121 ± 0.008	0.03 ± 0.01	0.04

[a] Alkyl-DHAP used as the substrate.
[b] Acyl-DHAP used as the substrate.
[c] Values represent the averages of determinations using two separate samples and did not vary by more than 10%.

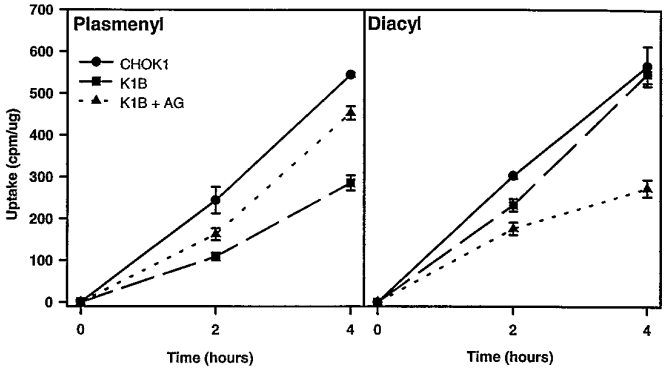

FIG. 5. **Ethanolamine labeling of phospholipids in CHO-K1 and FAA.K1B.** Cells were labeled with [1-^{3}H]ethanolamine at 37 °C followed by extraction and separation of the ethanolamine-labeled lipids as described under "Experimental Procedures." Two species, phosphatidylethanolamine and plasmenylethanolamine, contained >95% of the chloroform-soluble label. All values represent the average ± S.D. of three separate samples. ●, CHO-K1; ■, FAA.K1B; ▲, FAA.K1B supplemented with 20 μM *sn*-1-alkylglycerol.

established that the Kennedy-Weiss pathway is the primary pathway for phosphatidylcholine biosynthesis in CHO cells (35). We could, therefore, expect that a partial blockage of phosphatidic acid formation would have an impact upon phosphatidylcholine biosynthesis. Phosphatidylethanolamine, on the other hand, can be synthesized through the Kennedy-Weiss pathway, by head group exchange with phosphatidylserine (and phosphatidylcholine), or decarboxylation of phosphatidylserine (36). The Kennedy-Weiss pathway may not be important for phosphatidylethanolamine biosynthesis. In a CHO mutant strain that displayed a severe defect in diacylglycerol:ethanolamine phosphotransferase, part of the Kennedy-Weiss pathway, the biosynthesis of phosphatidylethanolamine was not greatly affected over an 8-h period, cell growth was relatively unaffected, and phosphatidylethanolamine levels were decreased by only 19% (37). However, CHO mutants, defective in head group exchange activity, showed a marked decrease in phosphatidylethanolamine levels (38, 39).

If acyl/alkyl-DHAP reductase is important for diacyl glycerolipid synthesis we might have expected to see increased levels of monoacylglycerol in the mutants, similar to the increased levels of alkylglycerol, However monoacylglycerol levels remained relatively unaffected. This may be due to the susceptibility of monoacylglycerols to degradation by lipases that are unable to cleave the ether bond but are active on ester linkages. Other laboratories have noted the relative metabolic stability of 1-alkylglycerol (40) and 1-alkyl-2-acylglycerol (41) when compared with 1-monoacylglycerol and diacylglycerol.

Surprisingly, the decrease in phospholipid biosynthesis did not appear to affect the growth of FAA.K1B (data not shown) or the ability of these cells to synthesize DNA and protein (Table VI). Also, the phospholipid content was only slightly affected. The mutant cells appear to have, at least partially, compensated for the biosynthetic lesion by decreasing phospholipid turnover (Fig. 4*B*). Decreased phospholipid turnover has been reported in other phospholipid biosynthesis mutants (38, 42). To some extent, the cells may have also been able to utilize phospholipid from the medium. Conditionally lethal mutants, displaying severe decreases in the biosynthesis of phosphatidylcholine and phosphatidylserine, could be rescued by supplementation of the medium with the affected phospholipids (38, 39, 43). Enough phospholipid may be found in the serum used to supplement the medium to compensate for a partial loss in phospholipid biosynthesis in FAA.K1B. We have observed that FAA.K1B is unable to grow in serum-free medium, but we have been unable to rescue this cell line with lipid supplementation.[2] We cannot, therefore, identify any lipid or lipids as the crucial ingredient in the serum.

TABLE VI

Protein and DNA synthesis in CHO-K1 and FAA.K1B

Protein and DNA synthesis were monitored by measuring the incorporation of [^{35}S]methionine and [^{3}H]thymidine, respectively, into trichloroacetic acid (TCA)-insoluble material as described under "Experimental Procedures." All values represent the average ± S.D. of three samples.

	Incorporation of label into TCA-insoluble material	
	[^{35}S]Methionine	[^{3}H]Thymidine
	cpm/mg protein	
CHO-K1	12.6 ± 0.4	2.89 ± 0.70
FAA.K1B	11.9 ± 1.6	3.55 ± 0.25

FAA.K1B is the first animal cell mutant to be described that is defective in a step in diacyl glycerolipid biosynthesis prior to the formation of phosphatidate, and it is defective in the first common step in the pathways for ether-linked and diacyl glycerolipid biosynthesis. Mutants such as these should help to examine the relationship between these two classes of lipid.

REFERENCES

1. Hajra, A. K. (1995) *Prog. Lipid Res.* **34,** 343–364
2. Datta, S. C., Ghosh, M. K., and Hajra, A. K. (1990) *J. Biol. Chem.* **265,** 8268–8274
3. Manning, R., and Brindley, D. N. (1972) *Biochem. J.* **130,** 1003–1012
4. Pollock, R. J., Hajra, A. K., and Agranoff, B. W. (1975) *Biochim. Biophys. Acta* **380,** 421–435
5. Rognstad, R., Clark, D. G., and Katz, J. (1974) *Biochem. J.* **140,** 249–251
6. Greenspan, M. D., Schroeder, E. A., and Yudkovitz, J. B. (1982) *Biochim. Biophys. Acta* **710,** 15–22
7. Declerq, P. E., Haagsman, H. P., Van Veldhoven, P., Debeer, L. J., Van Golde, L. M. G., and Mannaerts, G. P. (1984) *J. Biol. Chem.* **259,** 9064–9075
8. Hajra, A. K., Burke, C. L., and Jones, C. L. (1979) *J. Biol. Chem.* **254,** 10896–10900
9. Ghosh, M. K., and Hajra, A. K. (1986) *Arch. Biochem. Biophys.* **245,** 523–530
10. Datta, N. S., Wilson, G. N., and Hajra, A. K. (1984) *N. Engl. J. Med.* **311,** 1080–1083

[2] R. A. Zoeller, unpublished data.

11. Webber, K. O., Datta, N. S., and Hajra, A. K. (1987) *Arch. Biochem. Biophys.* **254,** 611–620
12. Nagan, N., Hajra, A. K., Das, A. K., Moser, H. W., Moser, A., Lazarow, P., Purdue, P. E., and Zoeller, R. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4475–4480
13. Zoeller, R. A., and Raetz, C. R. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5170–5174
14. Davis, P. A., and Hajra, A. K. (1981) *Arch. Biochem. Biophys.* **211,** 20–29
15. Corey, E. J., and Suggs, J. W. (1975) *Tetrahedron Lett.* **31,** 2647–2650
16. Schlossman, D. M., and Bell, R. M. (1976) *J. Biol. Chem.* **251,** 5738–5744
17. Hajra, A. K., and Burke, C. (1978) *J. Neurochem.* **31,** 125–134
18. Hajra, A. K., Datta, S. C., and Ghosh, M. K. (1992) *Methods Enzymol.* **209,** 402–412
19. Hajra, A. K., Saraswathi, T. V., and Das, A. K. (1983) *Chem. Phys. Lipids* **33,** 179–193
20. James, P. F., and Zoeller, R. A. (1997) *J. Biol. Chem.* **272,** 23532–23539
21. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
23. Esko, J. D., and Raetz, C. R. H. (1980) *J. Biol. Chem.* **255,** 4474–4480
24. Owens, K. (1966) *Biochem. J.* **100,** 354–361
25. Rouser, G., Siakotos, A. M., and Fleischer, S. (1966) *Lipids* **1,** 85–86
26. Wykle, R. L., and Snyder, F. (1970) *J. Biol. Chem.* **245,** 3047–3058
27. Myher, J. J., and Kuksis, A. (1989) *J. Chromatogr.* **471,** 187–204
28. Warne, T. R., Buchanan, F. G., and Robinson, M. (1995) *J. Biol. Chem.* **270,** 11147–11154
29. Jones, C. L., and Hajra, A. K. (1980) *J. Biol. Chem.* **255,** 8289–8295
30. LaBelle, E. F., Jr., and Hajra, A. K. (1974) *J. Biol. Chem.* **249,** 6936–6944
31. Morand, O. H., Zoeller, R. A., and Raetz, C. R. H. (1988) *J. Biol. Chem.* **263,** 11597–11606
32. Mosley, S. T., Goldstein, J. L., Brown, M. S., Falck, J. R., and Anderson, R. G. W. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 5717–5721
33. Snyder, F., Lee, T. C., and Wykle, R. L. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., ed) Vol. 2, pp. 1–58, Plenum Publishing Corp., New York
34. Zoeller, R. A., Rangaswamy, S., Herscovitz, H., Rizzo, W. B., Hajra, A. K., Das, A. K., Moser, H. W., Moser, A., Lazarow, P. B., and Santos, M. J. (1992) *J. Biol. Chem.* **267,** 8299–8306
35. Esko, J. D., and Raetz, C. R. H. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5192–5196
36. Kuge, O., Nishijima, M., and Akamatsu, Y. (1991) *J. Biol. Chem.* **266,** 24184–24189
37. Polokoff, M. A., Wing, D. C., and Raetz, C. R. H. (1981) *J. Biol. Chem.* **256,** 7687–7690
38. Voelker, D. R., and Frasier, J. L. (1986) *J. Biol. Chem.* **261,** 1002–1008
39. Kuge, O., Nishijima, M., and Akamatsu, Y. (1985) *Proc. Natl. Acad Sci. U. S. A.* **82,** 1926–1930
40. Rock, C. O., and Snyder, F. (1974) *J. Biol. Chem.* **249,** 5382–5387
41. Ford, D. A., and Gross, R. W. (1990) *J. Biol. Chem.* **265,** 12280–12286
42. Nishijima, M., Kuge, O., Maeda, M., Nakano, A., and Akamatsu, Y. (1984) *J. Biol. Chem.* **259,** 7101–7108
43. Esko, J. D., Nishijima, M., and Raetz, C. R. H. (1982) *Proc. Natl. Acad Sci. U. S. A.* **79,** 1698–1702