Synthesis of regiospecifically labeled [^{18}O]glycolic acid and [^{18}O]acyldihydroxyacetone phosphate

Dorothy M. Peterson, Rodolfo A. Martinez, Neera Satsangi, Susan T. Weintraub, Philip L. Stotter, and Samuel J. Friedberg

Department of Medicine, Division of Endocrinology and Metabolism and the Department of Pathology, University of Texas Health Science Center, and Division of Earth and Physical Sciences, University of Texas at San Antonio, San Antonio, Texas 78284

Abstract Methods are detailed for the preparation of [2-^{18}O]glycolate from chloroacetic acid and for the direct conversion of these intermediates to regiospecifically labeled [2-^{18}O]-2-*O*-acylglycolic acids containing approximately 90% ^{18}O at the C-*O*-acyl bond. Methods are also detailed for optimization of reaction conditions and yields for each synthetic step in previously published methods for the preparation of 1-*O*-acyldihydroxyacetone-3-*O*-phosphate (DHAP) from acyloxyacetic acid (i.e., 2-*O*-acylglycolic acid), where acyl is tetradecanoyl, hexadecanoyl, or heptadecanoyl. The optimized reaction conditions generate 1-*O*-acyl DHAP in its acid form, both in high overall yield and in high purity, without requiring a final chromatographic purification of the product, 1-*O*-acyl DHAP. Combining these new methods, efficient and facile preparations of regiospecifically labeled [1-^{18}O]-1-*O*-hexadecanoyl DHAP and [1-^{18}O]-1-*O*-heptadecanoyl DHAP have now been demonstrated, in which approximately 90% ^{18}O is specifically located only at the C-*O*-acyl position. Some mechanistic postulates are offered to account for the optimized yields, regioselectivities, and high ^{18}O incorporation which are observed in the reactions we have employed to generate 1-*O*-acyl DHAP from glycolate intermediates.—**Peterson, D. M., R. A. Martinez, N. Satsangi, S. T. Weintraub, P. L. Stotter, and S. J. Friedberg.** Synthesis of regiospecifically labeled [^{18}O]glycolic acid and [^{18}O]acyldihydroxyacetone phosphate. *J. Lipid Res.* 1988. **29:** 94–101.

Supplementary key words ether lipids • mass spectrometry

Glycolic acid, the substrate of photorespiration, can be used for the chemical synthesis of 1-*O*-acyl-dihydroxyacetone-3-*O*-phosphate (acyl DHAP), an obligatory biosynthetic precursor of ether lipids (1, 2). Glycolic acid or its glycolate salts can be made chemically from chloroacetic or other haloacetic acids by heating in water with or without base (3). We previously reported a modest yield preparation of glycolic acid containing 84.5% ^{18}O in the C-2 alcohol group (4); this regiospecifically labeled glycolic acid was converted (4) to mono-[^{18}O]acyl DHAP (having ^{18}O at the C-O-acyl position), using well known synthetic procedures (5, 6). In these procedures however, modest yields and purification methods associated with several individual steps (5, 6) have, in our hands, often proved unsatisfactory and/or nonreproducible. Consequently, we undertook the optimization of yields and improved isolation/purification techniques for each of the transformations in the preparation of pure 1-*O*-acyl DHAP (in its acid form) from acyloxyacetic acid. We also sought an isolation method that would avoid chromatographic purification of the final phospholipid product. As a result of these studies, we now describe suitable improvements for the general preparation of 1-(*O*)-acyl DHAP.

The ^{18}O-labeled acyl DHAP that we had previously prepared was used to study specific aspects of the biochemical mechanism of the ether lipid bond formation (4). In some subsequent preparations, our glycolic acid contained approximately only 50% ^{18}O at the desired position; this material was not sufficiently enriched in ^{18}O for planned biochemical studies requiring mass spectrometric analysis. We surmised that the problem of low ^{18}O incorporation was likely the result of several competing reactions that did not effectively distinguish ^{18}O of the medium and ^{16}O of the reagent COOH. Possibilities included rapid internal hydroxylation via a reactive α-lactone intermediate or formation of oligomeric (primarily dimeric) ester intermediates that were subsequently saponified to monomer in the basic medium. We have now addressed these problems, and detail here several sets of conditions appropriate for reproducible formation of ^{18}O-monolabeled glycolate products in satisfactory yield, containing ^{18}O with an isotopic enrichment of 85–93% at the desired C-2 position. These [^{18}O]glycolates have been converted to regiospecifically labeled [^{18}O]acyl DHAP by an improved synthetic sequence.

Abbreviations: DHAP, dihydroxyacetone phosphate; GLC-MS, gas-liquid chromatography-mass spectrometry; BSTFA, bis-(trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; FAB, fast atom bomdardment mass spectrometry; 16:0 acyl DHAP, hexadecanoyl dihydroxyacetone phosphate; 17:0 acyl DHAP, heptadecanoyl dihydroxyacetone phosphate; TMS, trimethylsilyl ether; TLC, thin-layer chromatography.

MATERIALS

Chloroacetic acid was obtained from Sigma (St. Louis, MO) and was recrystallized from benzene prior to use. ^{18}O-labeled water (98%) was obtained from Cambridge Isotope Laboratories (Woburn, MA). BSTFA [*bis*(trimethylsilyl) trifluoroacetamide] containing 1% TMCS (trimethylchlorosilane) was obtained from Pierce Chemical Co. (Rockford, IL). Glycolic acid (Eastman Kodak Co., Rochester, NY) was dried ($MgSO_4$) and subsequently recrystallized using ethyl ether. Oxalyl chloride (99 + %) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Tetradecanoyl, hexadecanoyl, and heptadecanoyl chlorides were freshly prepared from the corresponding purified fatty acids (Aldrich Chemical Co.) using oxalyl chloride or were obtained, respectively, from Aldrich Chemical Co. and Nu Chek Prep (Elysian, MN). Analytical and preparative thin-layer chromatography plates (silica gel, E. Merck) were obtained from Brinkman (Westbury, NY). Dry column chromatography was performed on silica gel G (Type G, dry column grade, Sigma Chemical Co.)

Dry methylene chloride was prepared by distillation of reagent grade methylene chloride from phosphorus pentoxide and was subsequently stored over 4A Molecular Sieves. Dry 1,4-dioxane was prepared by passing reagent grade 1,4-dioxane through neutral alumina (Brockman I, 80 g/100 ml of dioxane). Dry pyridine was prepared by stirring reagent grade pyridine over potassium hydroxide for 24 hr, followed by distillation from barium oxide.

EXPERIMENTAL

Melting points of all solid compounds were determined in open capillary tubes using a Thomas Hoover melting point apparatus. Those reported are uncorrected; only salient melting point data have been included for comparison with previous reports. IR spectra of all compounds were recorded on a Beckman IR 4220 or Perkin-Elmer 467 spectrophotometer to confirm purity and structure; unexceptional IR data have not been included. HNMR spectra were obtained for all compounds as solutions in $CDCL_3$ and/or CF_3CO_2D (TFA-d), using a General Electric QE300 or Varian EM390 spectrometer with tetramethylsilane as internal standard; selected spectra are reported for reference compounds with chemical shift values of salient resonances expressed in ppm relative to standard; spin-spin coupling patterns are described as follows: (s), singlet; (d), doublet; (t), triplet; (q), quartet; and (m), multiplet; with coupling constants (J) in Hz. Homogeneity of all purified compounds was routinely checked by thin-layer chromatography (TLC) using silica gel.

Negative ion FAB analyses were performed on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS 2200 data system. The accelerating voltage was 3 kV and the source temperature was approximately 60°C. Xenon was employed with an Ion Tech saddle field atom gun operating at 8 kV. Samples were applied in chloroform–methanol 8:2 (v/v) to the copper probe tip and allowed to dry in air. Approximately 3 μl of thioglycerol was then added and the matrix material was mixed with the sample.

Analysis of the trimethylsilyl derivative of glycolic acid was accomplished by gas–liquid chromatography–mass spectrometry on a Hewlett-Packard model 5982 mass spectrometer, in combination with a Hewlett-Packard model 5933 data system. The ion source temperature was 180°C, and the electron energy was 70 eV. Gas chromatographic separation was effected by means of a 6 ft × 4 mm glass column packed with 3% OV17 on 100/120 mesh Gas Chrom Q (Applied Science), using helium as the carrier gas at 30 ml/min. The injector temperature was 250°C, and the column temperature was 100°C. Samples were introduced into the mass spectrometer through a glass jet separator, maintained at 250°C. Samples of chloroacetic acid were analyzed using the same mass spectrometer settings, with sample introduction by means of a direct insertion probe.

[^{18}O]Glycolic acid

Chloroacetic acid (600 mg) was heated in 98% [^{18}O]H_2O (6 ml) at 100°C in two equal portions in 5-ml Reacti-Vials (Pierce Chemical Co.) sealed with a Teflon-lined cap for 2 weeks in a heating block.

Five μl of the combined reaction mixtures was dried under nitrogen in a 1-ml vial and then under high vacuum for 10 min. BSTFA (100 μl) was added and the mixture was heated at 60°C with stirring for 15 min. Examination of the resulting trimethylsilyl derivative of glycolic acid by gas–liquid chromatography–mass spectrometry (GLC-MS) showed **(Fig. 1a)** that 58% of the molecules contained three ^{18}O atoms and that 32% contained two ^{18}O atoms.

Half of the [^{18}O]water was then removed by distillation under reduced pressure at 50°C and recovered. Ordinary water (4 ml) was added and the volume was reduced to about 2 ml by vacuum distillation at 42°C. This procedure was then repeated three more times and the sample was finally reduced to 2 ml. In order to prevent polymerization catalyzed by residual acid, care was taken to avoid bringing the sample to dryness. The glycolic acid was diluted with 6 ml of ordinary water and stirred at room temperature for 4 hr to exchange the ^{18}O of the glycolic acid carboxyl group. One equivalent of sodium hydroxide was added (6.38 ml of 1 N NaOH) and the reaction mixture was dried in vacuo at 40°C. Repeat ex-

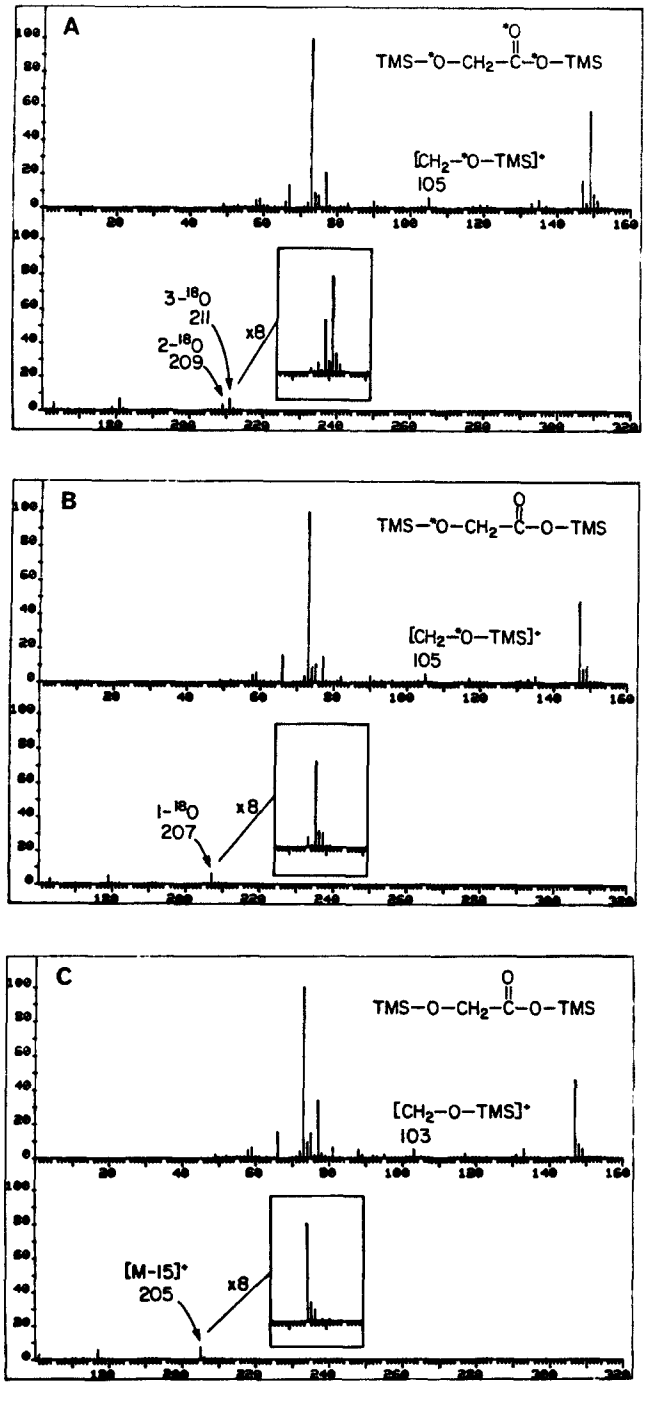


Fig. 1. Electron impact (70eV) mass spectra of the bis-trimethylsilyl derivative of glycolic acid. Samples were introduced into the mass spectrometer after elution from a gas chromatograph, as described in Methods. A, derived from the reaction of chloroacetic acid and [^{18}O]H_2O (98 mol%) after a 2-week incubation at 100°C; B, following several cycles of acid-catalyzed equilibration of sample A in H_2O with natural isotopic abundances of oxygen; C, derived from standard glycolic acid.

amination of the trimethylsilyl derivative by GLC-MS after neutralization with NaOH showed that the glycolic acid contained approximately 90% ^{18}O at C-2 (Fig 1b). The material derived in this fashion was used in the subsequent synthesis of acyloxyacetyl chloride.

[^{18}O] Heptadecanoyl dihydroxyacetone phosphate

[2-^{18}O]Glycolic acid was converted to heptadecanoyl dihydroxyacetone phosphate by the methods of Schlenk, Lamp, and De Haas (5) and Hajra and Agranoff (6). Prior removal of sodium chloride from the glycolic acid reaction mixture was found to be unnecessary in the initial synthesis of heptadecanoxy acetic acid. The intermediate product, heptadecanoxy acetic acid, was analyzed directly by mass spectrometry and was shown to contain at least 90% ^{18}O at the C-O-C bond. Otherwise the various reactions were carried out as originally described (5, 6). **Fig. 2** shows the negative ion fast atom bombardment (FAB) mass spectra of heptadecanoyl [^{18}O]DHAP and the hexadecanoyl ^{16}O analog. The major ion seen for each species is the $[M-1]^-$. Thus, for 16:0[^{16}O]acyl DHAP, the expected ion at m/z 407 is seen. For 17:0[^{18}O]acyl DHAP, an ion at m/z 423 is observed, an increase of 16 mass units from 16:0[^{16}O]acyl DHAP. This confirms the presence of one ^{18}O in the final 17:0 acyl DHAP. By comparison with unlabeled acyl DHAP, the total ^{18}O content was calculated to be greater than 90%.

Although the product obtained served our experimental needs, the yield in this reaction starting from heptadecanoxy acetic acid was only 6%. Consequently means were sought and then found to improve the methods of Schlenk et al. (5) and Hajra and Agranoff (6) to procure various acyl DHAPs in greater yield. Accordingly, the following modification was worked out using hexadecanoyl DHAP as a prototype.

Hexadecanoxyacetyl chloride

Hexadecanoxyacetic acid was obtained from freshly prepared hexadecanoyl chloride and glycolic acid as reported (5), except that total yields after isolation and purification were improved to >95% by combining the recrystallized product (from hexane) with additional material derived from the mother liquors and purified by TLC on silica gel using hexane-ethyl ether-acetic acid 50:50:1 (v/v/v) for development (R_f 0.35).

The purified acid (0.53 g, 0.0017 mol) was dissolved in methylene chloride (70 ml) and cooled to 0°C; oxalyl chloride (0.86 g, 0.0068 mol) was added to the cold solution and the reaction was allowed to warm to room temperature and then stirred for 2 hr. Solvent was removed in vacuo, affording hexadecanoxyacetyl chloride (0.56 g, 100%) as a pale yellow oil. The material was shown to be >95% pure and satisfactory for use in the next step, and for NMR and GLC-MS.

NMR ($CDCl_3$: 0.86 (t, J = 6Hz, 3H, $C\underline{H}_3$), 1.25 (broad m rising to sharp s, 24 H, C-4 to C-15 fatty acyl $C\underline{H}_2$), 1.63 (m, 2H, β-$C\underline{H}_2$), 2.41 (t, J = 7.5, 2H, α-$C\underline{H}_2$), 2.90 (s, 2H, $OC\underline{H}_2$-C = 0).

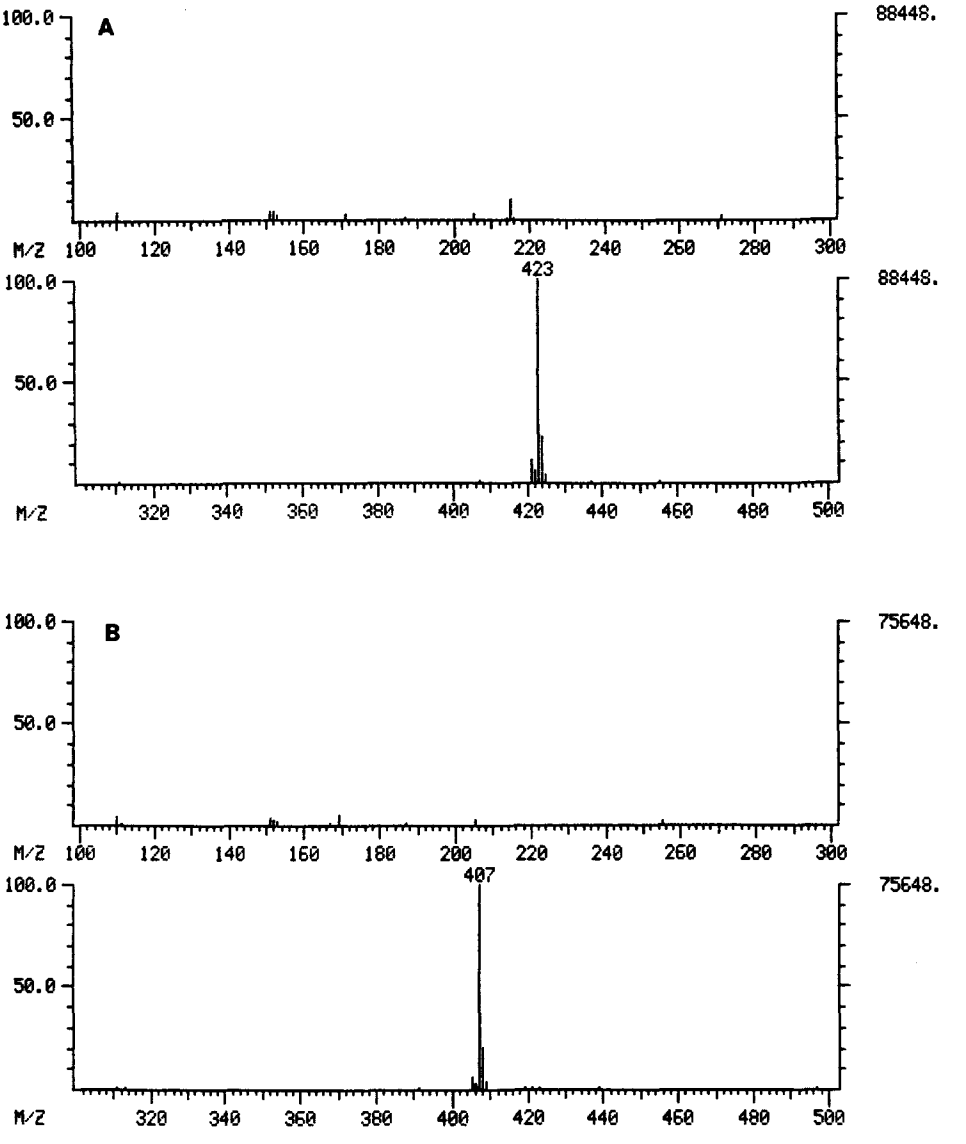


Fig. 2. Negative ion fast atom bombardment mass spectra of : A, heptadecanoyl-[^{18}O]DHAP and B, hexadecanoyl [^{16}O]DHAP. The contributions from the thioglycerol matrix have been subtracted.

Hexadecanoxyacetyldiazomethane (1-palmitoxy-3-diazoacetone

Hexadecanoxyacetyl chloride (0.56 g, 0.0017 mol) was dissolved in anhydrous ether (30 ml) and this solution was added dropwise to a cooled solution (0°C) of excess freshly prepared anhydrous diazomethane (0.28 g, 0.0068 mol) and triethylamine (0.17 g, 0.0017 mol) in ether (50 ml). After dropwise addition was complete, the solution was stirred for an additional 2 hr at 0°C. The resulting solution was evaporated to one-half its original volume under a stream of nitrogen, in order to remove the excess diazomethane. The ethereal suspension was partitioned between ether/brine to remove triethylammonium chloride and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated in vacuo. The residual pale yellow solid (0.56 g, 99%) contained less than 5% (reported (5) as approximately 30%) of a less polar impurity (TLC: ether–hexane 1:1 (v/v), R_f 0.6). The impurity was separated by preparative TLC and identified as 3-chloro-1-palmitoxyacetone, both by its spectral properties and by combustion analysis). Because simple recrystallization of the crude diazoketone did not readily afford product completely free of the chloroketone impurity, purification was routinely effected by dry column chromatography using ether–hexane 1:1 (v/v). After isolation, the purified diazoketone was subjected to high vacuum at 40°C for 24 hr to remove residual solvent, affording 0.52 g (95%, reported (5) as 63%) of solid product, mp 57–59°C, which was homogeneous by TLC

(ether-hexane 1:1 (v/v), R_f 0.3). Diazoketone prepared and purified in high yield by this method was more pure than, but otherwise spectrally (NMR and IR) and chromatographically (TLC) identical to, material prepared previously without modification of the reported procedure (5). The chromatographically purified diazoketone could be stored for extended periods or used directly in the next step. Repeated recrystallization from hexane followed by extended exposure to high vacuum raised the melting point to 59–60°C (reported (5) 58–59°C) and afforded a sample suitable for combustion analysis.

NMR ($CDCl_3$): 0.86 (t, J = 6Hz, 3H, $C\underline{H}_3$), 1.23 (broad m rising to sharp s, 24H, C-4 to C-15 fatty acyl $C\underline{H}_2$), 1.63 (m, 2H, β-$C\underline{H}_2$), 2.41 (t, J = 7.5 2H, α-$C\underline{H}_2$), 4.6 (s, 2H, $OC\underline{H}$-C=0), 5.5 (s, 1H, $C\underline{H}$ =N_2); calc. for $C_{19}H_{35}N_2O_3$ C, 67.22; H, 10.39; N, 8.25; found C, 67.40; H, 10.41; N, 8.40.

1-0-Hexadecanoyl-dihydroxyacetone-3-O-phosphoric acid (hexadecanoyl DHAP)

Phosphoric acid (85 wt%, 0.51 g, 0.045 mol) was dissolved in dry dioxane (5 ml) and heated to 70°C; to this phosphoric acid/dioxane solution was added a second solution containing 1-palmitoxy-3-diazoacetone (0.5 g, 0.0015 mol) dissolved in dry dioxane (5 ml) and also heated to 70°C. The reaction mixture was stirred at 70°C (rapid initial nitrogen evolution) for 2 hr until all nitrogen evolution had ceased and no residual starting material could be detected as monitored by TLC (ether-hexane 1:1 (v/v), R_f 0.45). The reaction mixture was cooled to room temperature and poured into ether (150 ml); the ethereal solution was washed well with water (3 × 50 ml), dried ($NaSO_4$) only until the solution began to cloud (initiation of phospholipid precipitation), rapidly filtered, and evaporated in vacuo to give a residue which proved to be a mixture of only two materials by TLC: nonpolar palmitoyldihydroxyacetone (ether-hexane 1:1 (v/v), R_f 0.3) and polar palmitoyl DHAP (ether-hexane 1:1 (v/v), R_f 0.0; and chloroform-methanol-acetic acid-water 50:25:7:1 (v/v/v/v), R_f 0.7). No other TLC spots were detectable using a variety of visualization techniques, e.g., I_2, sulfuric acid charring, and Dittmer and Lester phosphate spray (7). The mixture (0.70 g) was separated by addition of methylene chloride (7 ml) and subsequent agitation of the resulting slurry while hexane (21 ml) was added slowly; this suspension of palmitoyl DHAP was stirred for 1 hr, centrifuged for 15 min, and separated by decantation. The solids were twice resuspended in methylene chloride-hexane 3:1 (v/v), 12 ml), agitated, centrifuged, and isolated by decantation, as before. By TLC, the organic decantate showed a single spot (ether-hexane 1:1 (v/v), R_f 0.3). The solid palmitoyl DHAP was subjected to high vacuum for 24 hr to remove residual volatiles, at which time 0.53 g of product was collected. This material was shown to be homogenous and identical to an authentic sample, by TLC (ether-hexane 1:1 (v/v), R_f 0.0; and chloroform-methanol-acetic acid-water 50:25:7:1 (v/v/v/v), R_f 0.7). By NMR, the product contained no observable hydrogen-bound water or ethers.

NMR ($CDCl_3$TFA-d): 0.88 (t, J = 6Hz, 3H, $C\underline{H}_3$), 1.29 (broad m rising to sharp s, 24H, C-4 to C-15 fatty acyl CH_2), 1.68 (m, 2H, β-$C\underline{H}_2$), 2.55 (t, J = 7.5, 2H, α-$C\underline{H}_2$), 4.98 (d, J = 8.6, 2H, $POC\underline{H}_2$-C = O), 5.05 (s, 2H, $OC\underline{H}_2$-C = O), variable (2H, exchangeable $PO(O\underline{H}_2)$.

Tetradecanoyl and heptadecanoyl DHAP were prepared in the same manner and with similar yields. When ^{18}O incorporation was required, the final isotope enrichment was essentially equivalent to the isotope enrichment of the starting [2-^{18}O]glycolic acid. Spectral and chromatographic properties showed predicted differences.

Studies on the reaction conditions necessary for obtaining optimum yields of [^{18}O]glycolic acid

Base-catalyzed synthesis of [^{18}O]glycolic acid without preequilibration in [^{18}O]water. Chloroacetic acid (10 mg) was dissolved in [^{18}O]H_2O (100 μl). Metallic sodium (2.6 mg) was added and the reaction mixture was heated in a vial sealed with a Teflon-lined cap for 3 days at 100°C GLC-MS of the TMS derivative of glycolic acid showed that direct treatment of chloroacetic acid in [^{18}O]water with base resulted in the formation of glycolic acid containing approximately 50% ^{18}O in the primary alcohol group.

Base-catalyzed synthesis of [^{18}O]glycolic acid with preequilibration in [^{18}O]water. Chloroacetic acid (12 mg) was dissolved in [^{18}O]water (120 μl) and left at room temperature. The reaction mixture was monitored periodically by direct probe mass spectrometry. After 1 week, the product was chloroacetic acid containing more than 85% ^{18}O in the carboxyl oxygens **(Fig. 3)**. Metallic sodium (3 mg) was added and the reaction mixture was heated at 90°C for 1 week. Heating at higher temperatures apparently caused polymerization of the product. Subsequent GLC-MS of the TMS derivative of the resulting glycolic acid revealed that 87% contained three ^{18}O atoms and that 11% contained two ^{18}O atoms. The [^{18}O]water was evaporated under a stream of nitrogen at room temperature. Ordinary water (120 μl) was added, and the reaction mixture was stirred overnight at room temperature. Under these conditions the carboxyl oxygens did not exchange. Accordingly, the water was again removed and 0.1 N hydrochloric acid (120 μl) was added. After 24 hr it was found that 16% had retained three ^{18}O atoms. The reaction mixture was again evaporated under a stream of nitrogen and 0.1N HCl (96 μl) was added. After 2 more days of acid treatment, none of the glycolic acid molecules contained three ^{18}O atoms, 2.5% contained two ^{18}O atoms, and 90% contained one ^{18}O atom in the alcohol group.

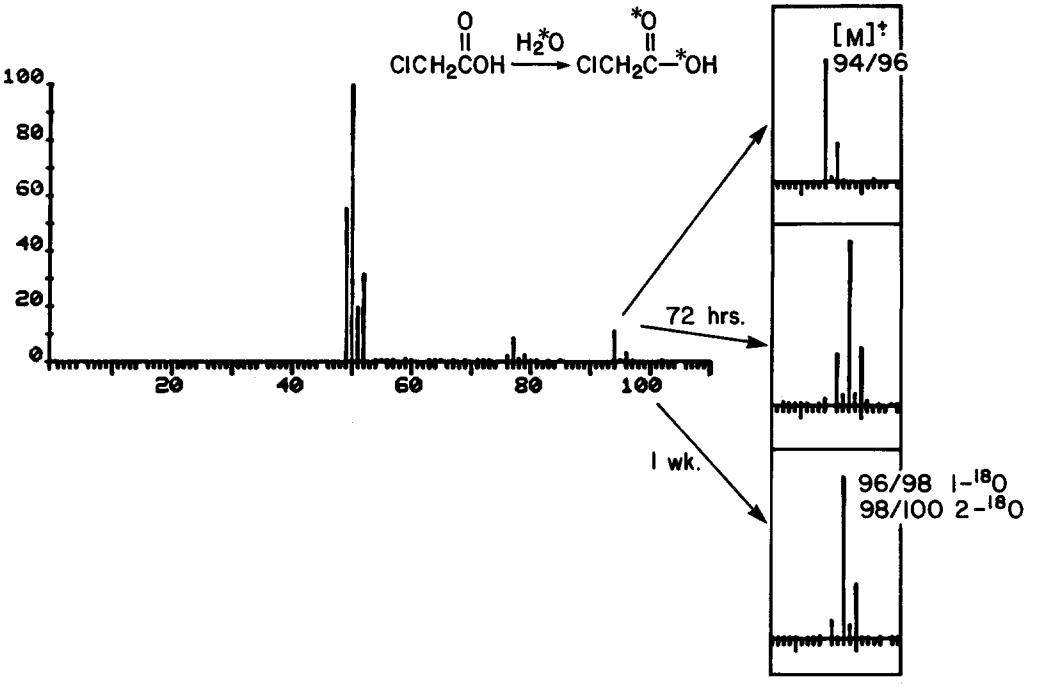


Fig. 3. Electron impact (70 eV) mass spectra of chloroacetic acid. The expanded insert shows the molecular ion cluster of the initial sample (top), after 72 hr of incubation in [^{18}O]H_2O (middle), and after 1 week of incubation (bottom). Sample introduction was by means of a direct insertion probe. Volatilization occurred at approximately 40°C.

DISCUSSION

Previously reported preparations (5, 6) of 1-*O*-acyl dihydroxyacetone phosphate (acyl DHAP) and of the corresponding regiospecifically monolabeled 1-[^{18}O] acyl DHAP (4) have, in our hands, suffered from a lack of reproducibility both in yields of isolated products and in our own % ^{18}O incorporation. In addition, it seemed apparent to us that the best yields reported (5, 6) for a number of steps in the synthetic sequence and isolation/purification had never been optimized. We have therefore undertaken a study of the optimization of yields and reproducibility for each step in the preparation, purification, and isolation of unlabeled 1-*O*-hexadecanoyl DHAP. We have also determined satisfactory methods for generating reproducibly high percentage incorporation of ^{18}O into glycolic acid and/or glycolate salts (from chloroacetic acid) for efficient preparation of ^{18}O-labeled hexadecanoyl and heptadecanoyl DHAP.

By combining crystallization and chromatography of mother liquors, total yields of acyloxyacetic acetic acids from glycolic acid were improved. Conversion of these acids to their acid chlorides was also improved by employing a suitable solvent which allowed the oxalyl chloride/carboxylic acid reaction to proceed at much lower temperatures than had been reported (5, 6). More importantly, the transformation of these acid chlorides to the corresponding diazoketones has been improved from 60% to >90% yield, by isolating and characterizing the by-product of the reaction and adjusting conditions to avoid production of this by-product. The by-product produced in 30% yield in the standard preparation (5, 6) was identified by NMR and combustion analysis to be the corresponding chloromethyl ketone (1-*O*-acyloxy-3-chloroacetone). Thus, as anticipated, inverse addition of the diazoketone precursor (the acyloxyacetyl chloride) to an ethereal solution of fourfold *excess* of diazomethane and triethylamine (to facilitate deprotonation of the intermediate α-diazonium methyl ketone) reduced the amount of the by-product to less than 5% with corresponding increase of the yield of diazomethyl ketone (to 90–95% after chromatography). We have found that the purified 1-*O*-acyl-3-diazoacetones are suitable for cold storage under inert atmosphere, allowing large scale preparations and storage of these precursors to acyl DHAP.

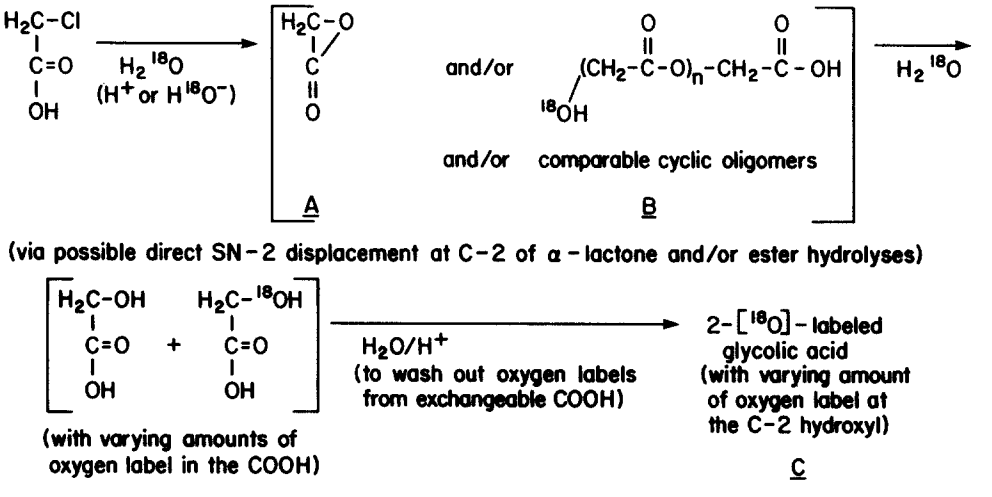
Conversion to the desired acyl DHAP species using purified diazoketone precursor was readily effected by modifying the reported procedure (6); thus, use of increased amounts of 85% phosphoric acid in hot dioxane with extended reaction times, until all precursor was consumed (as determined by TLC of the reaction mixture), afforded a clean product mixture containing only acyl DHAP and acyl DHA. This mixture could be readily separated *without chromatography* by extraction of the less polar impurity from the product mixture. The resulting insoluble acyl DHAP was obtained in 60% yield (in its

protonated form) directly by centrifugation/decantation and was shown both by NMR and by TLC to be a single, homogeneous compound. Of interest is the fact that the NMR spectrum of this material shows it is free of hydrogen-bonded water and other hydroxylic or ethereal materials.

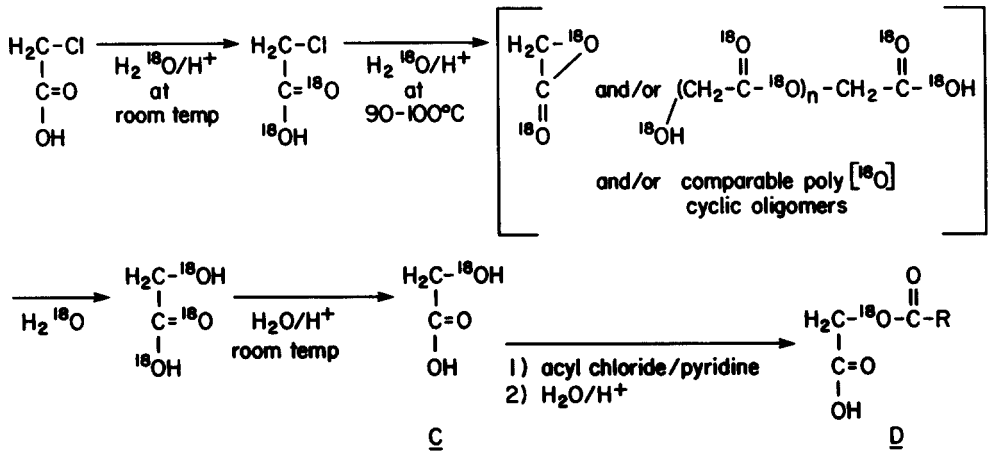
Application of this improved methodology to the preparation of regiospecifically labeled 1-[^{18}O]acyl DHAP allowed comparable yields and ease of isolation. The position of labeled oxygen was maintained. Moreover, the percentage incorporation of the label was shown by mass spectrometric techniques to correspond (within 5% deviation) to the amount of label at the C-2 hydroxyl group of labeled glycolic acid used as starting material for the synthetic sequence. As a result it remained only to find conditions for preparing this labeled glycolic acid in satisfactory yield and with reproducibly high percentage incorporation of ^{18}O. The first preparation (4) of [2-^{18}O]glycolic acid in these laboratories afforded a product containing 85% ^{18}O in the alcohol position. It was subsequently demonstrated that these initial results were observed because the starting chloroacetic acid had been dissolved in 99% [^{18}O]water and stored for several weeks before reaction to generate [^{18}O]glycolic acid. During this storage, fortuitous oxygen exchange of the oxygen atoms in the COOH occurred.

Subsequent attempts to reproduce these results without prior extended exposure of chloroacetic acid to [^{18}O]water led to glycolic acid containing only 50% of the oxygen label. Thus, as illustrated in **Scheme 1**, preparation of [2-^{18}O]glycolic acid (C) from chloroacetic acid by acid- or base-mediated solvolysis in excess $H_2{}^{18}O$ appeared to require formation of one or more intermediates such as α-lactones A and/or oligomeric polyesters B. Without prior exchange of the ^{16}O oxygens in the COOH group, the sources of oxygen at the C-2 hydroxyl in the product glycolic acid are both ^{16}O from COOH and ^{18}O from the medium (Scheme 1).

Since these observations appeared to be compatible with intermediate α-lactones and/or oligomeric polyesters in the conversion of chloroacetic acid to glycolic acid at all ranges of pH (as indicated in the preceding scheme), preparation of ^{18}O-labeled glycolic acid C (with *high* specific incorporation of the label) would appear to require formation of the α-lactone A and/or oligomeric polyesters in which *all* oxygen positions contained ^{18}O atoms (see Fig. 1). We found that, from chloroacetic acid in $H_2{}^{18}O$, solvolysis conditions that would allow prior COOH oxygen exchange with ^{18}O of the medium (acid-mediated, thermally accelerated exchange with subsequent solvolysis at elevated temperatures) generated glycolic acid containing multiple labeled oxygens as shown in **Scheme 2**. Subsequent removal of ^{18}O from the exchangeable COOH oxygens (by acid-catalyzed exchange with [^{16}O]water led to reproducibly satisfactory amounts (85–90%) of ^{18}O incorporation in the product [2-^{18}O]glycolic acid (C): thus, a solution of chloroacetic acid in $H_2{}^{18}O$ (99% ^{18}O) was heated to 100°C for 2 weeks; alternatively, ^{18}O was incorporated into the COOH of chloroacetic acid by exchange using excess $H_2{}^{18}O$ *at room temperature* (see Fig. 3); and then the resulting acid was warmed (acid- or base-mediated). Products of the several exchange/solvolysis/hydrolysis reaction variations (apparently formed via postulated intermediates A and/or B, Scheme 1) were multiple labeled glycolic acid; this material was then converted to 2-^{18}O-monolabeled C by acid-catalyzed exchange using $H_2{}^{16}O$/HCl (see Fig. 1). The regiospecific ^{18}O incorporation into monolabeled C observed for these procedures was 90% or greater. The reactions were monitored by GLC-MS by examining aliquots of the solvolyses



Scheme I



Scheme 2

and COOH equilibrations (Figs. 1 and 3). Final workup and isolation of C required adjustment of acidic solutions to pH 7 (or slightly higher) to avoid polymerization of the product glycolic acid. Acylation of C (containing glycolic acid and/or glycolate salts mixed with inorganic salts) could be directly effected in the normal fashion using freshly prepared fatty acid chlorides in pyridine to afford D in good yield **(Scheme 2)**.

^{18}O-Monolabeled hexadecanoyl DHAP and heptadecanoyl DHAP were prepared from monolabeled D . Mass spectral analysis using FAB (Fig. 2) proved that the product acyl DHAP in each case had maintained the ^{18}O. Moreover, we have previously shown (4) that mass spectral fragmentation (electron impact) of appropriate derivatives of ^{18}O-labeled acyl DHAP is completely consistent with the regiochemistry indicated in this report; in particular, the acylium fragment (RCO^+) derived from the fatty ester showed only the natural isotopic abundance of oxygen.

This investigation was supported by PHS grant 2 RO1 CA15047 awarded by the National Cancer Institute. Department of Health and Human Services, to Samuel J. Friedberg and by Robert A. Welch Foundation Grant AX637 and PHS Grant RR08194 to Philip L. Stotter. We gratefully acknowledge the assistance of Carolyn J. Cardenas, Thelma Barrios, and Nancy Garrett in the preparation of this manuscript. We also express our gratitude to The Southwest Foundation for Biomedical Research of San Antonio for the use of a Beckman IR 4220, a Perkin-Elmer 467 spectrophotometer, and a Varian EM390 HNMR spectrometer.

Manuscript received 11 May 1987 and in revised form 6 July 1987.

REFERENCES

1. Hajra, A. K. 1969. Biosynthesis of alkyl ether-containing lipid from dihydroxyacetone phosphate. *Biochem. Biophys. Res. Commun.* **37**: 486-492.

2. Wykle, R. L., C. Piantadosi, and F. Snyder. 1972. The role of acyl dihydroxyacetone phosphate, reduced nicotinamide adenine dinucleotide, and reduced nicotinamide adenine dinucleotide phosphate in the biosynthesis of O-alkyl glycerolipids by microsomal enzymes of Ehrlich ascites tumor. *J. Biol. Chem.* **247**: 2944-2948.

3. Eichloff, R. 1905. Über halogenirte Essigsäuren. *Justus Liebigs. Ann. Chem.* **342**: 115-123.

4. Friedberg, S. J., S. T. Weintraub, M. R. Singer, and R. C. Greene. 1983. The mechanism of ether bond formation in O-alkyl lipid synthesis in Ehrlich ascites tumor: unusual cleavage of the fatty acid moiety of acyl dihydroxyacetonephosphate. *J. Biol. Chem.* **258**: 136-142.

5. Schlenk, K. H., B. G. Lamp, and B. W. DeHaas. 1952. Syntheses of dihydroxyacetone and glycerides. *J. Am. Chem. Soc.* **74**: 2550-2552.

6. Hajra, A. K., and B. W. Agranoff. 1968. Acyl dihydroxyacetone phosphate. Characterization of a ^{32}P-labeled lipid from guinea pig liver mitochondria. *J. Biol. Chem.* **243**: 1617-1622.

7. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5**: 126-127.