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# ESTIMATION OF ACYLDIHYDROXYACETONE PHOSPHATE AND LYSOPHOSPHATIDATE IN ANIMAL TISSUES

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**Chemical and enzymatic methods have been developed to measure small quantities ($10^{-8}$–$10^{-10}$ mol) of acyldihydroxyacetone phosphate in animal tissues. Lipids extracted from tissue samples with acidic $CHCl_3$/methanol were subjected to solvent partitioning at two different pH values for partial purification of this keto-lipid from other lipids. This lipid was then estimated radiometrically either by chemical reduction with $NaB^3H_4$ or by enzymatic reduction with [4B-$^3$H]NADPH using a partially purified acyldihydroxyacetone-phosphate reductase (EC 1.1.1.101). Thin-layer chromatography revealed the presence of a number of $^3$H-labeled lipids in the $NaB^3H_4$-reduced product and further purification of the product was necessary to estimate the amount of acyl[2-$^3$H]glycerol 3-phosphate formed. The enzymatic reduction was very specific for acyl/alkyldihydroxyacetone phosphate. The amounts (nmol/g) of these keto-lipids estimated in different tissues by the enzymatic method were 10.06 ± 0.64 (guinea pig liver), 4.3 ± 0.15 (rat liver), 2.1 (rat testis), 1.5 (rad kidney) and 1.2 (rat brain). Monoacylglycerol 3-phosphate, i.e., lysophosphatidic acid, which was co-purified with acyldihydroxyacetone phosphate, was found to be present in relatively larger amounts in tissues. The amounts (nmol/g) of this lipid, estimated by enzymatically measuring the amounts of *sn*-glycerol 3-phosphate released after alkaline methanolysis of the partially purified lipid extracts, were 143 (guinea pig liver), 58 (rat liver), 53 (rat kidney) and 92 (rat brain). Stearic acid (18:0) was found to be the major (65%) fatty acid present in the lysophosphatidate purified from guinea pig liver.**

## Introduction

Acyldihydroxyacetone phosphate (acylDHAP) was discovered as a lipid rapidly labeled by [$\gamma$-$^{32}$P]ATP in guinea pig liver crude mitochondrial fraction [1,2] and was subsequently shown to be an important precursor of glycerolipids and glycerol-ether lipids [3–7]. Though its steady-state concentration in liver was estimated to be extremely low [8], the actual amounts of this lipid present in different tissues were never determined. While investigating the properties of this keto-lipid, it was found to be reduced by $NaBH_4$ to 1-acyl-(*rac*)-glycerol 3-phosphate (acylGro-3-*P*) under conditions where the ester bonds in lipids remained intact [2]. Using $^3$H-labeled $NaBH_4$ of high specific activity, very small amounts (less than $10^{-8}$ mol) of palmitoylDHAP could be quantitatively determined by measuring the amount of $^3$H-labeled lipid formed. This chemical method was employed to estimate the amounts of acylDHAP present in different tissues. An enzymatic method, simpler and more specific, was also developed. During the course of this investigation, it was discovered that

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Abbreviation: DHAP, dihydroxyacenone phosphate.

lysophosphatidate (acylGro-3-*P*), whose solubility and chromatographic properties are similar to those of acylDHAP, is present in relatively larger amounts than acylDHAP in different tissues. The analysis and estimation of these polar phospholipids in different tissues are reported here.

## Materials

Palmitoyl[$^{32}$P]DHAP was chemically synthesized from 1-*O*-palmitoyl 3-diazohydroxyacetone and $H_3{}^{32}PO_4$ (Amersham Corp., Arlington Heights, IL) as described before [9]. $NaB^3H_4$ and D-[1-$^3$H]glucose were obtained from Amersham (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively; $NaBH_4$, NADPH, hexokinase, D-glucose-6-phosphate dehydrogenase and DEAE-Sephacel were all purchased from Sigma Chemical Company (St. Louis, MO) and CL-Sepharose 6B was obtained from Pharmacia (Piscataway, NJ). E. Merck silica gel plates (VWR Scientific, Chicago, IL) were used for thin-layer chromatography. All solvents were of analytical grade. AG 50W-X4, H+ (100–200 mesh) was purchased from Bio-Rad Laboratories (Rockville Centre, NY) and washed with 2 M HCl before use. Rats (Sprague-Dawley) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and guinea pigs were purchased from Buckberg Lab Animals, Inc. (Tomkins Cove, NY).

## Methods

*Extraction and partial purification of acylDHAP by solvent partition*

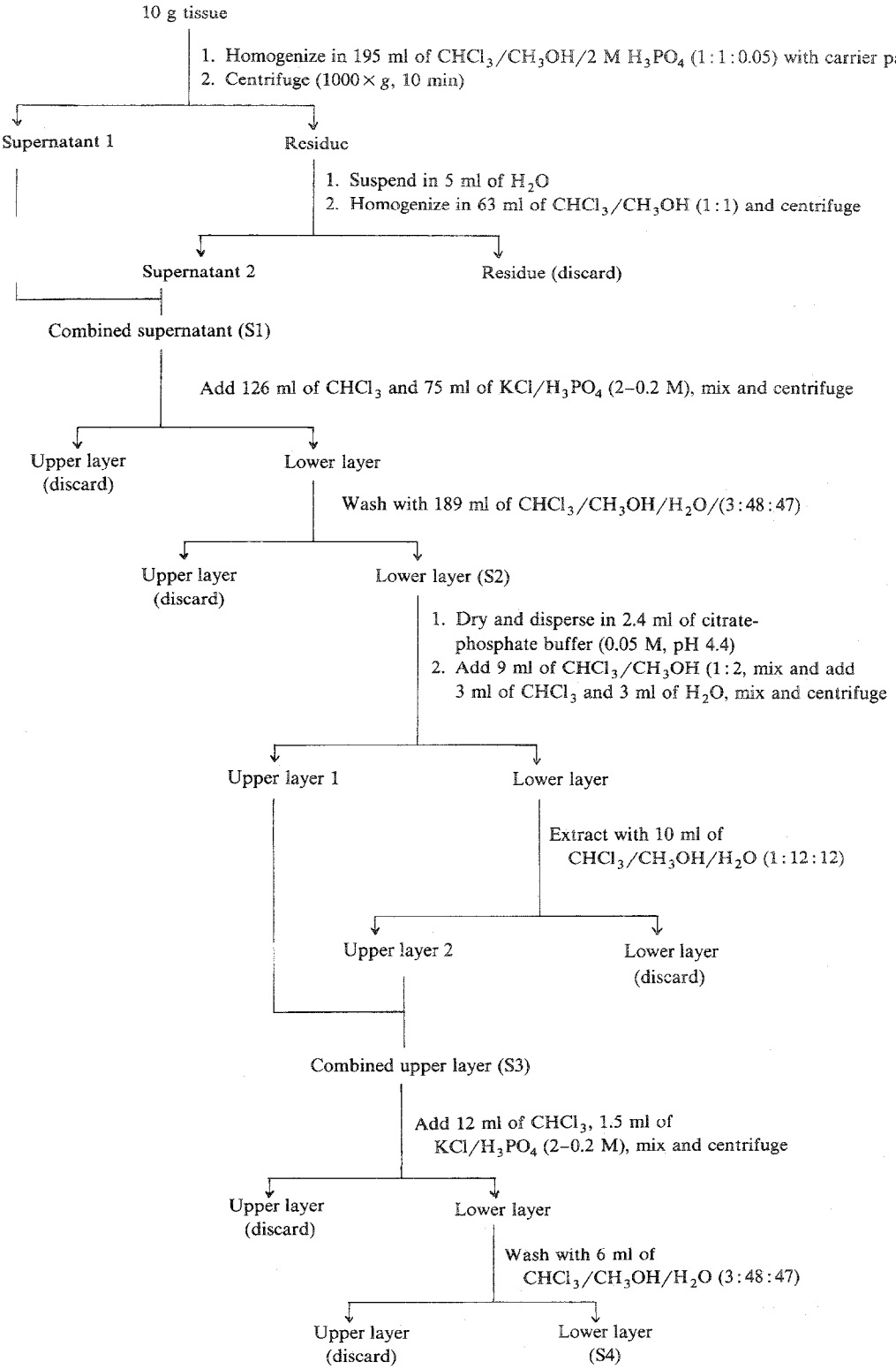
Lipids were extracted from tissues by an acidic solvent extraction method which was a modification of the method of Folch et al. [10]. Adult rats or guinea pigs were anesthetized with diethyl ether, the tissues were removed and immediately frozen in an isopentane/solid $CO_2$ bath and, if necessary, stored at −70°C. The frozen tissues were weighed, quickly ground into small pieces with a pre-chilled (−20°C) pestle and mortar and immediately homogenized in $CHCl_3$/methanol/$H_3PO_4$ (1 : 1 : 0.05) with a Polytron homogenizer as indicated in Scheme I. A trace amount (0.02–0.2 nmol, $(0.5–1.4) \cdot 10^5$ cpm/g of tissue) of carrier palmitoyl[$^{32}$P]DHAP was added to the homogenate to follow the recovery of acylDHAP at each stage of purification and also to verify the completeness of the reduction of acylDHAP in the extract by the chemical or enzymatic method. Details of the method of purification are described in Scheme I. Basically, the lipids were extracted with acidic $CHCl_3$/methanol and then partitioned between two phases under conditions where most of the acylDHAP went to the upper aqueous phase (pH 4.4), leaving most of the major tissue phospholipids in the lower phase [11]. During the partitioning process, the phases were mixed by vigorous vortexing (three to four times) and were then separated from each other by low-speed centrifugation ($1000 \times g$, 10 min). The acylDHAP was extracted back into another $CHCl_3$ phase under acidic (pH 1.5) conditions (Scheme I) and the amount present in the purified extract was estimated, either chemically or enzymatically as described below.

*Solubilization and partial purification of acyl/alkylDHAP: NADPH reductase from guinea pig liver*

Partially purified soluble acyl/alkylDHAP: NADPH reductase was prepared from guinea pig liver light mitochondrial fraction by a method similar to that described previously for acyl/alkylDHAP : NADPH reductase and DHAP acyltransferase [12,13]. Essentially, it was found that during the purification of DHAP acyltransferase the reductase was also solubilized and copurified with acyltransferase up to the ammonium sulfate fractionation stage. The reductase was partially separated from the acyltransferase by chromatography on CL-Sepharose 6B. The acyltransferase peak was at fraction No. 18 and the reductase peak was at fraction No. 20 (see Fig. 1. of Ref 13). Fractions No. 19–22 were pooled together and used for the enzymatic reduction of acylDHAP (see later). A summary of the results of the purification method is shown in Table I.

*Preparation and purification of [4B-$^3$H]NADPH by DEAE-Sephacel chromatography*

[4B-$^3$H]NADPH was prepared from D-[1-$^3$H]glucose [14] and purified by a modification of the method of Pastore and Friedkin [15]. The modification consists of using a high-pH buffer for




Scheme I. Extraction and purification of acylDHAP by solvent partition.

TABLE I

PURIFICATION OF ACYL/ALKYL-DHAP : NADPH OXIDOREDUCTASE FROM GUINEA PIG LIVER

The enzyme was purified from guinea pig liver light mitochondrial fraction by a method similar to that described previously [13]. To summarize: guinea pig liver light mitochondrial (peroxisomal) fraction was subjected to osmotic shock and the membrane-bound enzyme was solubilized with sodium-Cholate (0.2%) containing KCl (1 M). The soluble enzyme, after fractionation with ammonium sulfate, was subjected to gel filtration and the fractions containing the reductase were pooled as described in the text. The final yield of the enzyme was 4.8% of the initial light mitochondrial fraction. Purification factors are shown in parentheses.

| Fraction | Acyl/alkylDHAP reductase (nmol/min per mg protein) |
|---|---|
| Light mitochondria | 30.2 (1) |
| Membranes after osmotic shock | 56.8 (1.9) |
| KCl-cholate extract | 62.4 (2.0) |
| 20–35% $(NH_4)_2SO_4$ fraction | 90.4 (3.0) |
| CL-Sepharose 6B chromatography (pooled fractions) | 255.2 (8.4) |

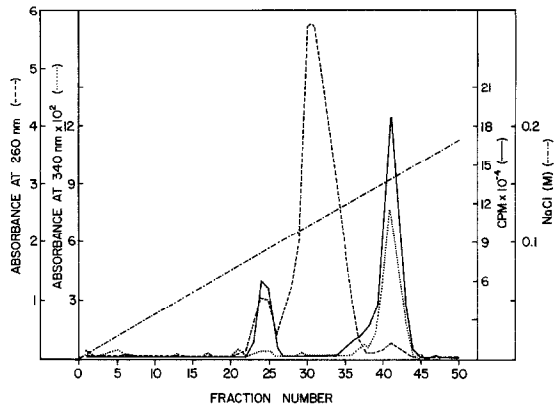



Fig. 1. Purification of [4B-$^3$H]NADPH by DEAE-Sephacel chromatography. Diluted incubation mixture was put on a DEAE-Sephacel column (7×1 cm) which was equilibrated with 0.01 M triethanolamine-HCl buffer (pH 8.5) at 4°C. The column was eluted with a linear gradient of NaCl in the same buffer and 5-ml fractions were collected. The radioactivity present in aliquots (10 $\mu$l) of each fraction (———) along with the absorbance at 260 nm (- - - - - -) and 340 nm (·····) were determined.

the enzymatic synthesis of the labeled NADPH and using DEAE-Sephacel instead of DEAE-cellulose to purify the NADPH. It was found that the high pH stabilized the NADPH formed, thus increasing the yield. DEAE-Sephacel is superior to DEAE-cellulose with respect to the time needed for chromatography and the resolution of the various components.

The incubation mixture contained D-[1-$^3$H]glucose (0.2 mM, 0.5 mCi), triethanolamine-HCl buffer (18 mM, pH 8.5), $MgCl_2$ (5 mM), ATP (10 mM, pH 7), D-glucose-6-phosphate dehydrogenase (50 $\mu$g), hexokinase (35 $\mu$g) and $NADP^+$ (1 mM) in a total volume of 1 ml. After incubation at room temperature (25°C) for 1 h, the mixture was diluted to 30 ml with $H_2O$ and loaded on to the DEAE-Sephacel column. Elution was done at 4°C with a linear gradient of 0–0.3 M NaCl as described in Fig. 1. The fractions containing [$^3$H]NADPH, as monitored by the absorbance at 340 nm (fractions No. 40–43), were pooled together, divided into 1-ml aliquots and lyophilyzed in a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, NY, Model No. SVC 100H-115). The dry [$^3$H]NADPH was stored at −70°C over desiccants and, when needed, was dissolved in water and used immediately.

*Estimation of acyl/alkylDHAP using the chemical method*

A stock solution of $NaB^3H_4$ (1 mCi/20 $\mu$mol) was prepared in 0.5 M NaOH by diluting $NaB^3H_4$ (23 mCi/$\mu$mol) with non-radioactive $NaBH_4$ and stored at −70°C. Before each chemical reduction, a small portion of the $NaB^3H_4$ solution was diluted to make the final alkali concentration 0.025 M. Total radioactivity in $NaB^3H_4$ was determined by incubating it with an excess of aqueous dihydroxyacetone at room temperature for 2 h and then counting the resulting [$^3$H]glycerol. The amount of acid-unstable radioactivity (actual amount of $B^3H_4^-$) was also determined by treating an aliquot of $NaBH_4$ with excess HCl [16]. The actual amount of labeled $NaBH_4$ present was calculated from these results. When the specific activity of $NaB^3H_4$ was estimated by adding it to an excess of [$^{32}$P]DHAP of known ($^{32}$P) specific activity and measuring the amount of [2-$^3$H,$^{32}$P]glycerophosphate formed (by high-voltage electrophoresis [1]), it was found to be 36.3 $\mu$Ci/$\mu$mol.

The reduction of the lipids with $NaB^3H_4$ was carried out at pH 7.6 as follows. Known amounts of acyl or alkylDHAP (0–20 nmol) or aliquots of liver lipid extracts (containing unknown amounts

of acyl/alkylDHAP) in $CHCl_3$ were placed in test-tubes and the solvents evaporated off under $N_2$. 200 $\mu$l of absolute ethanol, freshly distilled over $NaBH_4$, were added to the residues and sonicated to dissolve the substrates. 100 $\mu$l of Tris-HCl (0.3 M, pH 7.6) were then added and mixed by sonication. The reaction was started with 100 $\mu$l of $NaB^3H_4$ (2 mM, $3.2 \cdot 10^4$ cpm/nmol) and the mixture was incubated at 37°C for 2 h. The reaction was terminated by adding 3 ml of $CHCl_3$/methanol (2 : 1) followed by 0.6 ml of 1 M HCl to decompose the excess $NaBH_4$. After mixing and centrifuging, the upper layer was removed and the lower layer was washed with 1.5 ml of $CHCl_3$/methanol/water (3 : 48 : 37) [10]. An aliquot of the washed lower layer was dried and the radioactivity was determined by liquid scintillation spectrometry [14]. The efficiency of $^3H$-counting (Beckman LS-133 spectrometer) was 40%.

*Estimation of acyl / alkylDHAP using the enzymatic method*

The enzymatic reduction method was a modification of the procedure described by Labelle and Hajra [14]. Different amounts of acyl or alkylDHAP (0–20 nmol) or unknown amounts of lipid extracts in $CHCl_3$ were placed into a number of test-tubes. To each, 20 $\mu$l of Tween 20 (10 mg/ml of $CHCl_3$) were added, mixed and the solvent was removed completely by blowing $N_2$. The residue was dispersed in 50 $\mu$l of 0.05 M Tris buffer (pH 7.5) by sonication and the rest of the ingredients were added. The incubation mixture contained potassium phosphate buffer (33 mM, pH 7.5), NaF (10 mM), $Na_4EDTA$ (0.7 mM), [4B-$^3$H]NADPH (0.07 mM, 500 cpm/nmol), Tween 20 (0.2 mg), lipid substrates (see above) and enzyme protein (7–8 $\mu$g of the partially purified enzyme) in a total volume of 600 $\mu$l. After 15 min at 37°C, the reduced labeled lipids were extracted under acidic conditions, washed and the radioactivity of the lipid was determined as described above.

*Other methods*

The labeled products were identified by thin-layer chromatography ($CHCl_3$/methanol/acetic acid/5% aq. sodium metabisulfite, 100 : 40 : 12 : 4) followed by radioautography. When necessary, the radioactive bands were scraped out and extracted three times with $CHCl_3$/methanol (1 : 1) containing HCl (0.1 M). The extracts were combined, half volume of water was added, mixed and centrifuged. The upper layer was removed and the lower layer was found to contain most (more than 90%) of the radioactive acylDHAP or lysophosphatidate. For $^3$H-radioautography, the developed plates were sprayed with EN$^3$HANCE (New England Nuclear), covered with an X-ray film (Kodak X-Omat RP film) and stored at −70°C for 7–14 days before developing the film. For direct determination of radioactivity in the labeled spots, the corresponding areas were scraped out and the silica gel was dispersed in a scintillation solvent containing BBS-3 and counted in a liquid scintillation spectrometer [14]. The alkaline methanolysis was done as described previously [2] and the radioactivity of aliquots of the upper phase (water-soluble products) and the lower phase was determined. The upper layer containing water-soluble material was then passed through a small column (0.5 × 3 cm) of AG 50W-X4, H+ (100–120 mesh). The eluate was dried using a Speed Vac concentrator and the residues were dissolved in a very small volume of water. Aliquots were used for enzymatic estimation of glycerophosphate and also for high-voltage paper electrophoresis at pH 1.5 [1,2]. After high-voltage electrophoresis, the area of the paper containing the radioactive spot due to glycerophosphate (identified by checking the carrier $^{32}$P-labeled spot by autoradiography and also by using a standard sample side by side) was cut out into small pieces, the radioactive material extracted with 0.1 M HCl, and then counted using scintillation solvent containing BBS-3 [14]. Palmitoyl[$^{32}$P]glycerophosphate was prepared by the reduction of palmitoyl[$^{32}$P]DHAP with excess of $NaBH_4$, according to the procedure described above. The product was found to be pure by TLC (more than 98% radioactivity in acylglycerophosphate spot) using $CHCl_3$/methanol/acetic acid/5% aq. $NaHSO_3$ (100 : 40 : 12 : 4) as the solvent [17].

Methyl esters of fatty acids from lysophosphatidate were prepared by alkaline methanolysis [2,18] after the additions of a known amount of methyl heptadecanoate as internal standard and the esters were analyzed by gas chromatography.

A Hewlett-Packard gas chromatograph, Model No. 5710A, with dual glass-lined stainless steel analytical column (6′ × 0.125″, 15% Silar 10C on gas chrom R) and flame ionization detection was used. The temperature was varied between 150–220°C (2°C/min) and the carrier gas was $N_2$ at a flow rate of 70 ml/min. The methyl esters of different fatty acids were identified by comparing the retention times to that of a standard mixture of methyl esters of known composition and also by co-chromatography with the standards.

The concentration of a particular lipid (e.g., acylDHAP or lysophosphatidic acid) per g of tissue was calculated by measuring the amount of that lipid (or its hydrolytic product) present in an aliquot of the lipid extract and the fraction of the $^{32}P$ (added as a carrier $^{32}P$-labeled lipid to the tissue during initial homogenization, see Scheme I) recovered in that extract. Glycerophosphate was estimated using glycerophosphate dehydrogenase in hydrazine-containing buffer as described by Bublitz and Kennedy [19]. The total lipid phosphates were estimated by the method of Ames and Dubin [20]. Protein was determined either by the method of Lowry et al. [21] or by a modified Lowry procedure [22] using bovine serum albumin as standard.

## Results

*Optimum conditions for the reduction of palmitoyl (acyl)- and hexadecyl (alkyl)DHAP by $NaB^3H_4$*

$NaB^3H_4$ was employed to reduce the acylDHAP or alkylDHAP to the corresponding [2-$^3$H]glycerol 3-phosphate derivatives. The reduction of carbonyl compounds by $NaBH_4$ is generally carried out at high pH but, in the present method, Tris-HCl buffer (pH 7.5) was included for the reduction of these keto-lipids because acylDHAP is extremely alkali labile [2]. Ethanol was added to keep the lipids in solution. Under such conditions, the optimum concentration of $NaB^3H_4$ was 1–2 mM (20–40 molar excess over the substrates) (Fig. 2A) and the reaction was complete within 2 h at 37°C (Fig. 2B).

Using the optimum conditions (2 mM $NaB^3H_4$ at 37°C for 2 h), the amount of $^3$H-labeled lipids formed was found to be directly proportional to the amount of 1-*O*-palmitoylDHAP or 1-*O*-hexadecylDHAP used for the reduction within a range of 0–30 nmol (Fig. 3). Using palmitoyl[$^{32}$P] DHAP, the reduction was shown to be complete

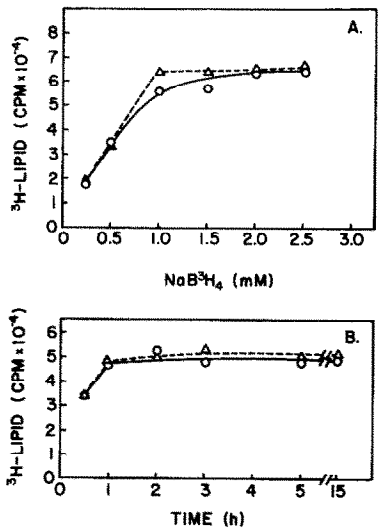



Fig. 2. Effect of increasing $NaB^3H_4$ concentration and the time-course of the reduction of acyl- and alkylDHAP. 20 nmol of palmitoyl DHAP (△- - -△) or hexadecylDHAP (○———○) were reduced either with varying concentrations of $NaB^3H_4$ for 2 h at 37°C (A) or with 2 mM $NaB^3H_4$ for different periods of time at 37°C (B) (see text for detailed procedure).

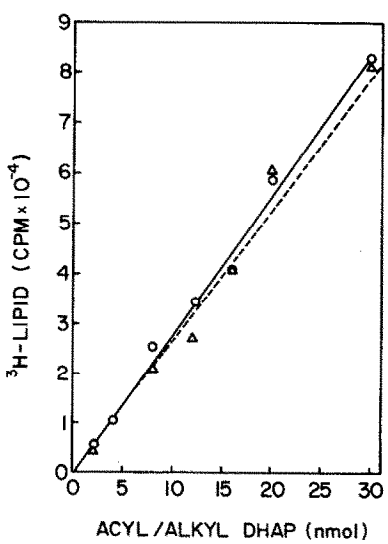



Fig. 3. Stoichiometry of the reduction of substrates with $NaB^3H_4$. Increasing amounts of synthetic palmitoylDHAP (△- - -△) or hexadecylDHAP (○———○) were reduced with $NaB^3H_4$ and the amounts of labeled lipids formed were determined as described in Methods.

by examining the product by thin-layer chromatography, using $CHCl_3$/ methanol/ acetic acid/5% aqueous sodium bisulfite as the solvent. On the chromatogram, only a single spot corresponding to lysophosphatidate ($R_F$ 033), well-separated from acylDHAP ($R_F$ 0.2), was seen. The specific activity of the $^3H$-labeled product, however, was found to be about 50% of the theoretical value of the radioactive $NaB^3H_4$ used, assuming four molecules of the carbonyl compound are reduced by one molecule of $NaBH_4$ [23].

*Extraction and purification of acylDHAP from tissues*

The acidic extraction and partition conditions described in Scheme I are found to be efficient for the near quantitative recovery of acylDHAP with the removal of most (more than 98%) of the other tissue phospholipids. For example, the recovery of carrier palmitoyl[$^{32}$P]DHAP from liver homogenate was 93 and 85% at Stages 2 and 4 (Scheme I), respectively, whereas the total phospholipids ($\mu$mol of phospholipid phosphorus per g of tissue) at those two stages (S2 and S4) were 40.5 and 0.66 in rat liver and 54.9 and 0.76 in guinea pig liver, respectively.

*Estimation of acylDHAP by reduction with $NaB^3H_4$*

$^3H$-labeled lipids were formed when the liver lipid extract containing acylDHAP was reduced with $NaB^3H_4$. Using a known amount of palmitoylDHAP as standard, the amount of $^3H$-labeled lipid was found to be about 480–520 nmol/g of guinea pig liver and 260 nmol/g of rat liver. However, when attempts were made to characterize the labeled lipid as acyl [2-$^3H$]Gro-3-*P* (reduced product of acylDHAP) by thin-layer chromatography, it was observed that a number of $^3H$-labeled spots, other than acyl Gro-3-*P*, were present on the chromatogram (Fig. 4). This indicated that, in the extract, other lipids were present which reacted with $NaB^3H_4$ to give $^3H$-labeled products.

The amount of acyl[2-$^3H$]Gro-3-*P* formed by the reduction of acylDHAP present in the liver lipid extract was determined by further purification of the $^3H$-labeled product. The spot corresponding to 1-acylGro-3-*P* (located by the presence of carrier $^{32}$P-labeled lipid on the chromatogram) was scraped out and the lipid was extracted. The labeled lipid (184 nmol of $^3H$/g liver) was then subjected to alkaline methanolysis and the water-soluble labeled [$^{32}$P,$^3H$]Gro-3-*P* was further purified by high-voltage paper electrophoresis at pH 1.5. The [$^{32}$P]Gro-3-*P* spot was localized by radioautography, the corresponding areas were extracted and the amount of $^3H$ present in the extracts was determined. The amount of this [$^3H$]Gro-3-*P* was found to be directly proportional to the amount of liver (up to 80 mg) originally used to extract the lipid (correlation coefficient 0.97). Using this extensive purification method, the amount of acylDHAP (measured by the

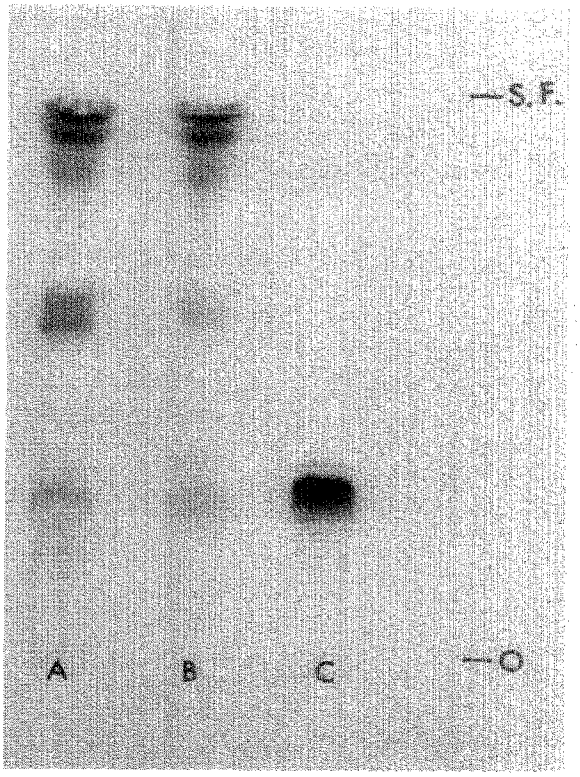



Fig. 4. Radioautogram of the $NaB^3H_4$-reduced lipids product after separation by TLC. In this particular experiment no carrier palmitoyl[$^{32}$P]DHAP was added during lipid extraction. A. Partially purified guinea pig liver lipid (stage 4 of Scheme I) after reduction with $NaB^3H_4$. B. Same as A except that rat liver was used. C. $NaB^3H_4$-reduced palmitoylDHAP (standard). The chromatogram was developed with $CHCl_3$/methanol/ acetic acid/aqueous bisulfite (5%) and the $^3H$-radioautograph was prepared as described in the Methods (O, origin; S.F., solvent front).

amount of [$^3$H]Gro-3-*P* obtained as described above) was calculated to be 10.6 nmol/g of guinea pig liver.

*Enzymatic estimation of acylDHAP*

The chemical method ($NaB^3H_4$ reduction) of estimation of acylDHAP as described above is a lengthy procedure because of extensive purification necessary to remove other labeled contaminants. Therefore, an alternative method, using specific enzymatic reduction of acylDHAP, was developed for the rapid estimation of this lipid in tissue samples. The enzymatic reduction method is similar to that described for the assay of the enzyme [14] but, to ensure complete reduction of the substrate (acyl- or alkylDHAP), excess enzyme and excess [4B-$^3$H]NADPH were used with limiting amounts of acylDHAP. Under such conditions palmitoyl- or hexadecylDHAP (up to 20 nmol) was found to be completely reduced to the corresponding [2-$^3$H]Gro-3-*P* derivatives and the labeled lipid formed is directly proportional (correlation coefficient 0.99) to the amount of acyl- or alkylDHAP used in the incubation mixture. The specific activity of the labeled lipid formed was, however, about 85% of the calculated specific activity of the labeled NADPH used for the reduction.

Applying this enzymatic reduction method to rat and guinea pig liver lipid extract, it was observed that $^3$H-labeled lipid formed is proportional (correlation coefficient 0.99) to the amount of lipid extracts used. Also, when examined by thin-layer chromatography, $^3$H-labeled lipid migrated as a single spot in two different chromatographic systems ($CHCl_3$/methanol/acetic acid/$H_2O$, 100 : 40 : 12 : 4, and $CHCl_3$/methanol/acetic acid/5% aqueous $NaHSO_3$, 100 : 40 : 12 : 4) with the same migration rate as lysophosphatidic acid (Fig. 5). The reduced $^3$H-labeled product also comigrated with the $NaBH_4$-reduced product of palmitoyl[$^{32}$P]DHAP, added as a carrier. When the labeled ($^{32}$P, $^3$H) lipid was subjected to alkaline methanolysis, all of the $^{32}$P and 75% of the $^3$H became water-soluble and, on high-voltage paper electrophoresis, comigrated with authentic glycerol 3-phosphate. These results indicate that only acylDHAP and alkylDHAP (alkali stable) present in the lipid extracts are specifically reduced by NADPH, catalyzed by the acyl/alkylDHAP reductase.

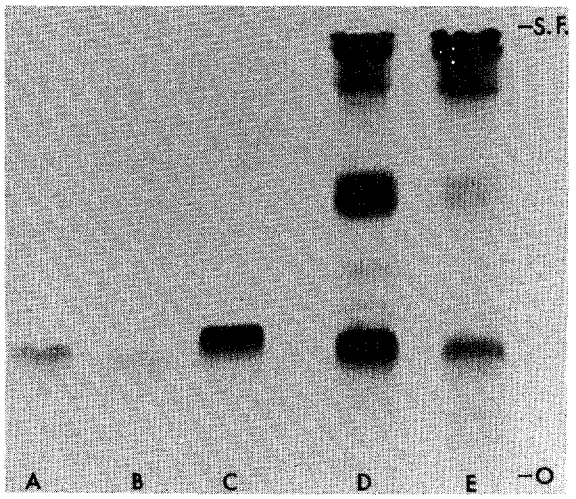



Fig. 5. Radioautogram of the TLC-separated products obtained by both enzymatic reduction using [4B-$^3$H]NADPH and the chemical reduction using $NaB^3H_4$ (shown side by side for comparison). No carrier palmitoyl[$^{32}$P]DHAP was added during lipid extraction. The chromatographic procedure was the same as described in Fig. 4 (O, origin; S.F. solvent front). A. Partially purified guinea pig liver lipid (stage 4 of Scheme I) after enzymatic reduction with [4B-$^3$H]NADPH. B. Same as A, except that rat liver was used. C. Enzymatically reduced palmitoylDHAP (standard) with [4B-$^3$H]NADPH. D. Same as A in Fig. 4. E. Same as B in Fig. 4.

*Estimation of acyl/alkylDHAP in other different tissues*

The enzymatic method, described above, was used to measure the concentration of acylDHAP present in different tissues of rat. The same procedure as described for guinea pig liver, i.e., lipid extraction with acidic $CHCl_3$/methanol and purification of acylDHAP by solvent partition (monitored by the added palmitoyl[$^{32}$P]DHAP) followed by enzymatic reduction with $^3$H-labeled NADPH, was used for different tissues and the results are presented in Table II. It is seen that small but reproducible amounts of acylDHAP are present in every tissue examined (Table II). Guinea pig liver contained the highest amount of acylDHAP (0.018% of total phospholipid, 1.32% of the purified lipid extract) followed by rat liver (0.01% of total phospholipid, 0.65% of lipid extract).

TABLE II

CONTENT OF ACYL/ALKYL-DHAP IN DIFFERENT TISSUES OF ADULT RAT AND IN LIVER OF ADULT GUINEA PIG

The amount of acylDHAP was measured by the enzymatic reduction of lipid extracts with [4B-$^3$H]NADPH as described in the text. The results are expressed as the amount per g of tissues after correction was made for the loss of acylDHAP during the extraction procedure (see Methods for detail).

| Tissues | Acyl/alkylDHAP (nmol/g of tissue) |
|---|---|
| Rat liver | $4.30 \pm 0.15$ [a] |
| Rat testis | 2.18, 2.12 [b] |
| Rat kidney | 1.39, 1.53 [b] |
| Rat brain | 1.20, 1.31 [b] |
| Guinea pig liver | $10.06 \pm 0.64$ [a] |

[a] Average of five experiments using livers from different animals on different days ± S.D.

[b] Results of two experiments.

*Estimation and analysis of lysophosphatidic acid in guinea pig liver*

During the course of the purification and estimated of acylDHAP, it became apparent that a number of other acidic phospholipids were present in tissues whose solubilities and chromatographic properties are similar to acylDHAP. Monoacylglycerol 3-phosphate, i.e., lysophosphatidic acid is one such lipid whose presence in the purified liver lipid extract was inferred when relatively larger amounts of glycerol 3-phosphate were found to be present in the alkaline methanolysate of the $NaB^3H_4$-treated lipid extract than could be accounted for by the amount of acylDHAP present in the liver. Experiments were undertaken to purify and measure the lysophosphatidate in guinea pig liver. The method as outlined below is basically the same as described for acylDHAP. A trace amount of carrier 1-acyl-(*rac*)-[$^{32}$P]Gro-3-*P* (0.1 nmol, $0.7 \cdot 10^5$ cpm/g of tissue) was added to the liver homogenate in $CHCl_3$/methanol/$H_3PO_4$ and the lipids were extracted and purified as described in Scheme I. The total recovery of the labeled lipid was 95.0%. The lipid was further purified by preparative thin-layer chromatography ($CHCl_3$/methanol/acetic acid/5% sodium bisulfite, 100 : 40 : 12 : 4) and the $^{32}$P-band was scraped out and the lipid was extracted with $CHCl_3$/methanol/HCl as described in Methods. After measuring the phosphorus content of this purified lipid extract it was calculated to be 237 nmol of phospholipid phosphorus per g of liver. However, when the amount of lysophosphatidate in this purified extract was measured by alkaline methanolysis [2] followed by the estimation of *sn*-glycerol 3-phosphate [19], a linear relationship was obtained with the increasing amount of extracts which was calculated to be 143 nmol/g of liver. To check whether this difference was due to the presence of inhibitors of glycerophosphate dehydrogenase in the extract, known amounts of *sn*-Gro-3-*P* were added to the sample hydrolysates, and the glycerophosphate in the resulting mixture was enzymatically determined. The increase in glycerophosphate corresponded exactly to the amount of glycerophosphate added. These results indicated that other phospholipids besides lysophosphatidate are present in this purified lipid extract. Therefore, the lysophosphatidate was further purified by another thin-layer chromatography using a basic solvent system ($CHCl_3$/methanol/8 M $NH_4OH$, 60 : 40 : 5). In this system lysophosphatidate migrated just above the origin ($R_F$ 0.07) and when the plate was sprayed with Molybdenum blue spray [24] two other phospholipid bands ($R_F$ values 0.20 and 0.37) were seen to be present on the chromatogram. The lysophosphatidate band was scraped out (before Molybdenum blue spraying) and the lipid was extracted as described before. When analyzed, this extensively purified lysophosphatidate gave a ratio of total phosphate/glycerophosphate of 1 : 1.01 (144 nmol/g vs. 142 nmol/g), indicating that lysophosphatidate is uncontaminated with any other phospholipid. The fatty acid composition of this purified lysophosphatidate was determined using methyl heptadecanoate as internal standard (Table III). The mole ratio of fatty acid (154 nmol/g) to glycerophosphate was found to be 1.08 : 1, a further indication that lysophosphatidate is the only lipid present in the final purified extract.

*Estimation of lysophosphatidate in different tissues*

The results presented above show that there is relatively much more lysophosphatidate than

TABLE III

FATTY ACID COMPOSITIONS OF GUINEA PIG LIVER LYSOPHOSPHATIDIC ACID

The methyl esters were prepared from the purified guinea pig liver lysophosphatidic acid (with the addition of methyl heptadecanoate as the internal standard) and were subjected to gas chromatographic analysis as described in the text. The average values of three gas chromatographic analyses are given here.

| Fatty acid | mol% |
|---|---|
| 14:0 | 3.1 |
| 16:0 | 9.2 |
| 18:0 | 65.9 |
| 18:1 | 5.7 |
| 18:2 | 14.3 |
| 22:0 (?) | 1.8 |

acylDHAP present in guinea pig liver. The lysophosphatidate concentrations in different tissues of the rat were determined by measuring the amount of *sn*-glycerol 3-phosphate liberated after alkaline methanolysis of the partially purified lipid extract (Stage 4 of Scheme I). The amount found was (in nmol/g tissue) 58.0 for liver, 53.2 for kidney and 91.6 for brain.

## Discussion

The results presented above show that acylDHAP is present in all tissues examined, albeit in extremely small quantities. Among the tissues, this lipid is present in highest amounts in guinea pig liver, where it was originally discovered [1]. The concentration of acylDHAP in guinea pig liver and in rat liver was found to be fairly constant in different animals at different periods of time (Table II). However, it is possible that the concentration may change under physiological conditions, such as during fasting and feeding or chronic administration of hypolipidemic drugs where the activity of DHAP acyltransferase is changed [25,26]. The steady-state concentration of acylDHAP, however, is very low in tissues such as in developing rat brain (Das and Hajra, unpublished data) and kidney, where DHAP acyltransferase activity is high [27,28]. This indicates that acylDHAP is quickly reduced by the reductase (acylDHAP: NADPH reductase) whose activity in tissues is much higher than that of DHAP acyltransferase [14]. The concentration of acylDHAP in different tissues is comparable to CDPdiacylglycerol, another active lipid metabolite [29]. It should also be pointed out here that part of the acylDHAP found in tissues is alkylDHAP, which could be measured as the amount of alkali-stable $^3$H-labeled formed after enzymatic reduction by [$^3$H]NADPH. Further work in this laboratory is directed towards measuring the amount of acylDHAP and alkylDHAP in different tissues under different physiological conditions.

$NaB^3H_4$ quantitatively reduced acylDHAP and it is shown here that this reduction can be utilized as a sensitive method for the estimation of acyl/alkylDHAP. However, the stoichiometry of radioactive precursor to product is not as theoretically predicted, which is probably due to the isotope effect ($^3$H vs. $^1$H), especially because $NaB^3H_4$ of high specific activity is diluted with non-radioactive $NaBH_4$ before use. This non-stoichiometric incorporation of radioactivity is seen when DHAP is used as described above or with D-glucose as reported by McLean et al. [16]. Under the conditions of the reduction, the ester bond of acylDHAP remained intact, as evidenced by the complete conversion of the carrier acyl[$^{32}$P]DHAP to acyl [2-$^3$H]Gro$^{32}P$. However, it is possible that some active esters, other than the acyl groups in acylDHAP or acylglycerophosphate, are reduced to long-chain alcohols by $NaB^3H_4$, as described by Nichols and Safford [30].

It is surprising to find that many $^3$H-labeled lipids were formed when the partially purified acidic lipid extract was reduced with $NaB^3H_4$. The identities of most of these lipids are unknown at present. However, one of these spots is probably due to long-chain acyl CoAs which formed labeled long-chain alcohols after reduction [31,32]. Others could be ketosteroids (glucouronide or sulfate derivatives?) which behaved like acidic lipids in this extraction process. The properties of some of these lipids are, however, very similar to acylDHAP (e.g., migrating on TLC with acylDHAP before reduction and with acylGro-3-*P* after reduction), which even formed a $^3$H-labeled water-soluble product after reduction with $NaB^3H_4$ and alkaline methanolysis. It is possible that some derivatives of acyl- or alkylDHAP or other minor keto-lipids

are present in tissues which have not yet been identified. Future investigations should be directed towards identifying these minor tissue lipids.

Unfortunately, because of the presence of other labeled lipids, extensive purification of the product was necessary to estimate the amount of acylDHAP present in the extract. This made the procedure lengthy and somewhat impractical. Also, because of the very small quantities of acylDHAP present in the extract which could not be freed easily from impurities, one of our objectives, i.e., determining the fatty acid composition of acylDHAP, could not be fulfilled. However, the development of the alternative enzymatic method made it relatively simple to estimate acylDHAP in the lipid extract. The main drawback of this method is the lack of a commercial source of labeled NADPH and the reductase. However, the enzyme can be partially purified in few steps and is stable for 3–4 weeks when stored at 4°C. We also found that the labeled NADPH need not be purified if it is used within 1 week of preparation. In such a case, the incubation mixture containing [4B-$^3$H]NADPH, prepared from D-[1-$^3$H]glucose (see above), was mixed with non-radioactive NADPH to the required specific activity and stored at 4°C (pH 8.5). Standard acylDHAP or alkylDHAP should be run each time when the amount of acylDHAP in lipid extract is determined enzymatically with [$^3$H]NADPH because there is a small isotope effect during enzymatic reduction which has been reported previously [14].

One interesting finding during this investigation is that tissues contain much more lysophosphatidate than acylDHAP. Though the presence of lysophosphatidic acid in tissues has been described by different workers [33,34], such a large amount (almost the same as that of phosphatidic acid) has not been reported before. This is probably because, during the extraction of tissue lipids, this lipid is lost because acidic extraction, as employed here, is generally not used. The estimation, by measuring *sn*-Gro-3-*P* formed after alkaline methanolysis, is specific because phosphatidic acid, the only other lipid which would generate Gro-3-*P* under such conditions, is not extracted by the procedure described above [11]. The position of the fatty acid at the glycerol moiety in this tissue lysophosphatidate is presently not known, but in all probability it is mostly at the C-1 position because it was found that 80% of the fatty acid is saturated (Table III).

Surprisingly, stearate is found to be the major (66%) fatty acid, with very little palmitate, present in the purified lysophosphatidate from guinea pig liver (Table III). A similar fatty acid distribution (62% stearate) was also observed in the lysophosphatidic acid isolated from rat liver (Das and Hajra unpublished data). This is quite in contrast to the fatty acid composition of liver phosphatidate where the fatty acid at C-1 is mostly palmitic (51%) with a smaller fraction of stearate (33%) [35]. Lysophosphatidate in tissues is biosynthesized in mitochondria and microsomes by acylation of *sn*-Gro-3-*P* and in peroxisomes by acylation of DHAP, followed by reduction. The acyltransferase which catalyzes the conversion of lysophosphatidate to phosphatidate is reported to be present only in microsomes [6]. The acylation of Gro-3-*P* in mitochondria and DHAP in peroxisomes preferentially occurs with palmitoyl-CoA [28,36] but the microsomal Gro-3-*P* acyltransferase is somewhat nonspecific [37]. Therefore, it is expected that biosynthetic lysophosphatidate should contain mostly palmitate and not stearate at the C-1 position of the Gro-3-*P* moiety. However, the fatty acid composition is also dependent on the composition of the tissue acyl-CoAs and it has been reported that stearoyl-CoA comprises about 72% of liver saturated fatty acyl-CoAs [32]. Alternatively, lysophosphatidate may form via breakdown of tissue lipids. One such process is described in platelets, where phosphoinositides are shown to be hydrolyzed to diacylglycerol which forms lysophosphatidate after phoshorylation and hydrolysis by phospholipase $A_2$ [38]. The lysophosphatidate formed via this cycle is expected to be enriched in stearate because the C-1 positions of phosphoinositides in different tissues have been shown to contain mostly (80%) stearic acid [39]. It would be interesting to study whether or not the amount of lysophosphatidate in tissues is increased by hormonal or neuronal stimulation which is known to stimulate the 'phosphatidylinositol cycle'. Future investigations should be directed towards determination of the amount and nature of lysophosphatidate present in tissues under different physiological conditions.

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