Quantitative micro determination and isolation of plasmalogen aldehydes as 2,4-dinitrophenylhydrazones

IRA KATZ and MARK KEENEY

Department of Dairy Science, University of Maryland, College Park, Maryland

SUMMARY A micro spectrophotometric procedure for the quantitative determination of plasmalogen aldehydes is described which utilizes simultaneous methanolysis and formation of 2,4-dinitrophenylhydrazones. After isolation of the hydrazones by thin-layer chromatography, the aldehydes can be regenerated, reduced, acetylated, and then analyzed by gas–liquid chromatography. Identification of the plasmalogen aldehydes obtained from rumen holotrich protozoa is described.

KEY WORDS plasmalogen aldehydes · determination · 2,4-dinitrophenylhydrazones · spectrophotometry · thin-layer chromatography · aldehyde · regeneration · reduction · composition · gas–liquid chromatography · ethanolamine glycerophosphatides · rumen protozoa

PROCEDURES FOR THE simultaneous methanolysis of fatty acid esters and the formation of DNP hydrazones from bound aldehydes, followed by column chromatography of the products, have been successfully applied to the quantitative determination of aldehydes in rumen bacteria (1). The approach is a modification of the Wittenberg method of fatty aldehyde determination (2), in which DNP hydrazine has been substituted for *p*-nitrophenyl hydrazine and anhydrous methanol has been substituted for 95% ethanol. The present report deals with micro-scale adaptation of the reactions and separation of the products by TLC. The isolated methyl esters and regenerated aldehydes can then be studied by GLC. Results of the analysis of some aldehyde-rich lipids from protozoa of the rumen are presented. A sample containing 5 μmoles of aldehyde can be conveniently carried through the whole procedure of aldehyde regeneration, reduction, acetylation, and GLC of the acetates. If one is interested only in the total aldehyde content, a sample containing < 1 μmole of aldehyde is sufficient for accurate spectrophotometric analysis.

Materials and Reagents. Carbonyl-free methanol was prepared by refluxing 500 ml of reagent grade methanol for 2 hr with 1 g of DNP hydrazine and 2 ml of 85% phosphoric acid. The methanol was distilled, and stored in a tightly stoppered amber bottle. The light yellow color in the distillate was ignored, as it was not extractable into hexane. Before use, 10–50 ml of the methanol was treated with $NaBH_4$, 2 mg/ml, and allowed to stand for 15 hr in a glass container fitted with a ground glass joint.

DNP hydrazine (Matheson, Coleman, & Bell, East Rutherford, N. J.) was purified by stirring 2.5 g of it for 5 min in 500 ml of boiling carbonyl-free hexane, prepared by H_2SO_4 scrubbing as described by Wishner and Keeney (3). The hexane was decanted, and the procedure was repeated a second time. The DNP hydrazine was collected by filtration through Whatman No. 1 filter paper on a Büchner funnel.

Approximately 0.5 N H_2SO_4 in methanol was prepared by adding 0.15 ml of concd H_2SO_4 to 10 ml of carbonyl-free methanol. The solution was allowed to stand for a minute to insure the complete destruction of any residual $NaBH_4$. DNP hydrazine was then dissolved in the acidic

Abbreviations: DNP, 2,4-dinitrophenyl; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DEAE, diethylaminoethyl.

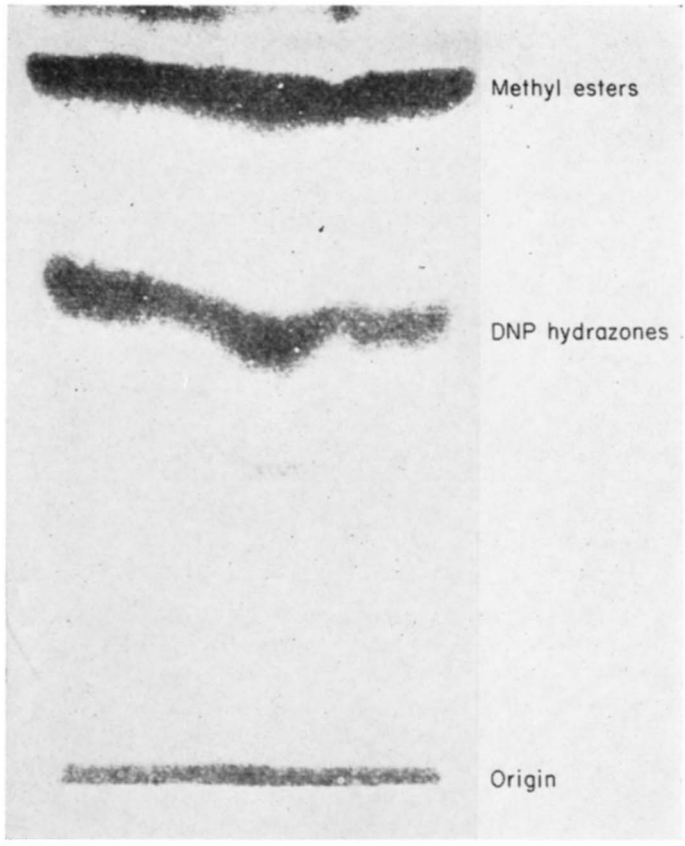

FIG. 1. Thin-layer chromatogram of DNP hydrazones and methyl esters from the ethanolamine glycerophosphatides obtained from rumen holotrich protozoa. Developing solvent: hexane–ether–acetic acid 85:1:52 (v/v/v). Detection method: sprayed with 50% H_2SO_4 and then charred at 200°.

methanol to a concentration of 13 mg/ml. As a precautionary procedure, a fresh solution was prepared every 3 days.

For $LiAlH_4$ reduction, 100 ml of freshly distilled, anhydrous, peroxide-free diethyl ether was stored over a few lumps of metallic sodium and decanted off as needed.

Levulinic acid solution, for the regeneration of aldehydes from DNP hydrazones, was prepared by adding 1 volume of N HCl to 9 volumes of levulinic acid, as described by Keeney (4).

For TLC, glass plates (20 × 20 cm) were coated with Silica Gel H (E. Merck AG, Darmstadt, Germany) to a thickness of about 500 μ. After drying, the plates were washed twice in chloroform–methanol–acetic acid 50:50:2 (v/v/v). The absorbent was activated before use at 110° for 2 hr.

Rumen holotrich protozoa were isolated by the glucose sedimentation procedure described by Heald and Oxford (5). The lipids were extracted from the wet packed cells with 20 volumes of chloroform–methanol 2:1. Forty liters of rumen digesta yielded 980 mg of protozoa lipid. A phosphatidyl ethanolamine fraction, rich in phosphatidal ethanolamine, was isolated from the lipids by chromatography on DEAE-cellulose (6). The phospholipid was chromatographically identical with that of a known sample of phosphatidyl ethanolamine (Applied Science Laboratories, State College, Pa.). In addition, its infrared spectrum was also identical, except for an additional absorbance at 6.0 μ which is attributable to the vinyl ether linkage (7).

In order to minimize losses by volatilization, solvents were carefully evaporated at 35° under a stream of nitrogen. The tubes were removed as the last trace of solvent evaporated.

PROCEDURE

Formation of DNP Hydrazones and Methyl Esters. A solution of up to 15 mg of phospholipid, containing a known amount of P, was evaporated to dryness in a 15 × 125 mm test tube. The sample was dissolved in 1 ml of the DNP hydrazine solution by shaking, and warming if necessary. The tubes were tightly capped and allowed to stand for 15 hr at room temperature. After the addition of 2 ml of water, the DNP hydrazones and methyl esters were extracted with two 5-ml portions of hexane. In some instances, slight centrifugation was necessary to remove

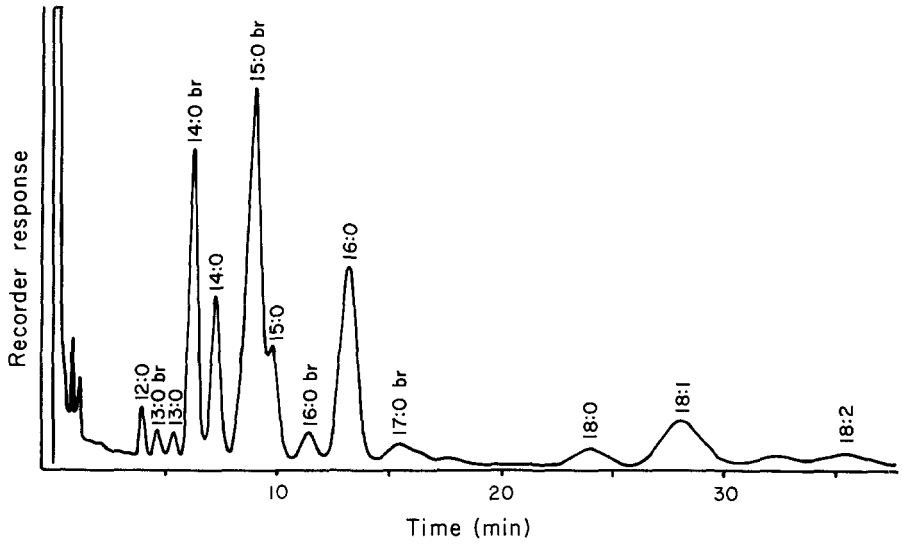

FIG. 2. Gas chromatogram of phosphatidal ethanolamine aldehydes (as alkyl acetates) obtained from rumen holotrich protozoa. Conditions: 6 ft × 4 mm stainless steel column packed with diethyleneglycol succinate polyester, 20% on 80–90 mesh Anakrom ABS; column temperature, 185°; preheater temperature, 250°; nitrogen inlet pressure, 12 psi. Identification of peaks as indicated.

any suspended DNP hydrazine from the hexane layer.

Quantification of DNP Hydrazones. The absorbance of the hexane solution of DNP hydrazones was determined at 338 mμ in a 1 cm cell against a blank prepared in the absence of lipid. The concentration was calculated from a molar absorptivity of 21,500 (8).

Separation and Isolation of DNP Hydrazones and Methyl Esters. The hexane extract was evaporated almost to dryness, and then transferred to a 10 × 75 mm test tube in two 1-ml portions of $CHCl_3$. The $CHCl_3$ was almost completely evaporated, and the remaining material was dissolved in 0.2 ml of $CHCl_3$. The entire solution was carefully streaked by means of a 50 μl syringe in a repeating dispenser (Brinkmann Instruments Inc., Westbury, N. Y.) across a 20 × 20 cm Silica Gel H plate. The plates were developed in hexane–ether–acetic acid 85:15:2. After drying, the plates were sprayed with an aqueous 0.001% solution of Rhodamine 6G, and then viewed under an ultraviolet lamp. The plates were allowed to dry again, and the desired areas were scraped into 12 × 250 mm glass columns plugged with glass wool. The DNP hydrazones and methyl esters were quantitatively eluted from the columns with 5 column volumes of ether. The methyl ester fraction may be analyzed by GLC if desired.

Regeneration and Reduction of the Fatty Aldehydes. The regeneration was accomplished by a slight modification of the levulinic acid procedure of Keeney (4). One milliliter of the levulinic acid–HCl solution and 1 ml of CH_2Cl_2 were added to the dry DNP hydrazones in a 15 × 125 mm test tube. The tube was immersed in a boiling water bath to a depth of 2 cm for 12 min. The solution was cooled, 2 ml of water was added, and the regenerated aldehydes were extracted with two 3-ml portions of hexane. The yellow color due to unregenerated aldehydes was ignored, as it did not interfere with the GLC analysis. After careful evaporation of the hexane, the residual aldehydes were immediately reduced to their corresponding alcohols by the addition of 1 ml of a 0.25% solution of $LiAlH_4$ in anhydrous ether (9). After 10 min at room temperature, 2 ml of water, 3 ml of ether, and 3–4 drops of 10 N H_2SO_4 were added. The mixture was extracted for 10–20 sec on a Vortex mixer (Scientific Industries Inc., Queens Village, N. Y.). The ether layer was removed and the extraction was repeated with 3 ml of ether. The combined ethereal extracts were passed through a 10 mm glass chromatographic column, containing 1 g of $MgSO_4$–Na_2CO_3 2:1 (w/w). The column was washed with 3–4 ml of ether to elute the alcohols completely. The alcohols were recovered after the evaporation of the ether.

Acetylation. The alcohols were acetylated in 3 ml of pyridine–acetic anhydride 2:1 for 15 min at 35°, as described by Farquhar (10). After the addition of 3 ml of water, the acetates were extracted with two 3-ml portions of *n*-pentane. The combined pentane extracts were shaken with 3 ml of N HCl, until the odor of pyridine could no longer be detected. After evaporation of the pentane, the residual acetates were gas-chromatographed.

RESULTS

The quantitative aspects of the simultaneous DNP hydrazone and methyl ester formation were examined by using

a model system of *n*-heptaldehyde semicarbazone and butter fat. When 10, 15, and 20 μmoles of heptaldehyde semicarbazone were treated under the described conditions in the presence of 4 mg of butter fat, yields of DNP hydrazone ranging from 94 to 100% of the theoretical were obtained. In addition, TLC of a portion of the hexane extract containing 10 μmoles of DNP hydrazone resulted in the recovery of 95% of the material applied to the plate. The methanolysis was presumably quantitative, as no evidence for the presence of mono-, di-, or triglyceride was obtained on the thin-layer plates.

The levulinic acid-HCl regeneration procedure was checked by determining the amount of unregenerated aldehyde (4). The regeneration of three samples containing 1.5, 6.3, and 20 μmoles of heptaldehyde DNP hydrazone resulted in a 97, 96, and 92% regeneration, respectively.

Analysis of Protozoa Ethanolamine Glycerophosphatides. Duplicate samples of protozoa phospholipid containing 13.5 μmoles of P were subjected to the described procedure. Aldehyde/P molar ratios of 0.49 and 0.52 were obtained. When a sample containing 7.1 μmoles of DNP hydrazones was subjected to TLC, 6.7 μmoles of long-chain aldehyde DNP hydrazones was isolated. The separation of the DNP hydrazones and methyl esters obtained from the protozoa ethanolamine glycerophosphatides is shown in Fig. 1.

The results were verified by cleaving the aldehydogenic moiety of the phospholipid by the acetic acid procedure of Gray (11) and separating the products by silicic acid column chromatography. A lysophosphatidyl ethanolamine P/total P ratio of 0.52 was obtained.

The aldehyde DNP hydrazones obtained by TLC were regenerated, reduced to their alcohols, and then acetylated. The acetates were gas-chromatographed on diethyleneglycol succinate polyester. Identification was based upon the relative retention time compared to standards prepared by the reduction and acetylation of authentic fatty acid methyl esters. Branched-chain fatty acids were obtained from rumen bacterial phospholipids, a rich source of these compounds (12). The identification of unsaturated esters was aided by hydrogenation. The chromatogram is illustrated in Fig. 2. In addition, 7 μmoles of dodecanal DNP hydrazone was carried through the procedure. GLC of the acetate yielded only one major peak, apart from a few peaks on the shoulder of the solvent peak.

DISCUSSION

The procedure as outlined cannot be applied to lipids that are insoluble in methanol. This disadvantage may be overcome by the addition, when necessary, of up to 1 ml of CH_2Cl_2. However, if the extraction of excess DNP hydrazine is to be prevented, the CH_2Cl_2 must then be evaporated prior to the hexane extraction. During the evaporation the volume is kept at approximately 1 ml by the addition of methanol. Even when excess reagent appears in the extract, it is easily removed as it is virtually insoluble in hexane, and remains at the origin upon TLC.

The reaction time of 15 hr was selected in order to insure a complete methanolysis. If one is interested only in the aldehyde concentration, a time of 1 hr is sufficient. If desired, the methanolysis reaction may be driven to completion by refluxing the CH_3OH solution for 1–2 hr.

We have noted that in general, when pure, freshly isolated phospholipids are assayed for aldehydogenic material, the hydrazones in the initial hexane extraction consist almost entirely of long-chain ($>C_{11}$) aldehyde DNP hydrazones. When crude phospholipid extracts, or older partially oxidized samples are assayed, DNP hydrazones other than those from long-chain aldehydes are obtained in the hexane extract. The long-chain aldehyde concentration in such samples is determined by subjecting the hexane extract, or an aliquot of it, to TLC. The long-chain aldehyde concentration is then determined from the long-chain DNP hydrazones recovered from the plate. The contaminating DNP hydrazones usually migrate more slowly than the long-chain aldehyde DNP hydrazones.

The preparation and use of carbonyl-free methanol was prompted by the high blank values obtained with reagent grade methanol. The particular batch used for this work had an initial carbonyl concentration of 0.18 μmole/ml. After distillation from DNP hydrazine, this was reduced to 0.024 μmole/ml. The treatment with $NaBH_4$ further lowered it to 0.006 μmole/ml.

This is the first report of the presence of plasmalogens in the lipids of rumen holotrich protozoa. Because of the paucity of information about the lipid composition of these anaerobic organisms, we are extending our work to include a detailed characterization of these lipids.

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