Simplified procedures for hydrolysis or methanolysis of lipids

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CONVENTIONAL PROCEDURES for hydrolysis of lipids and quantitative recovery of the hydrolysis products are time-consuming and require relatively large samples. These deficiencies become particularly evident when large numbers of lipid samples are processed for analysis by gas-liquid chromatography (GLC). A considerable simplification could be achieved if the hydrolysis and the separation of the hydrolysis products were carried out in the same flask. Such a flask was introduced nearly 20 years ago by Entenman, Taurog, and Chaikoff (1), and consisted of an Erlenmeyer flask having a side tube that would retain the lower phase in the extraction process. We describe here a modified version of this flask, together with some modified cleavage procedures used in conjunction with it. These methods have proved advantageous, particularly in connection with GLC analysis.

Apparatus

The combination hydrolysis and extraction flask consists of a 50 ml Pyrex Erlenmeyer flask with standard taper joint (19/16 or 19/38), having a 5 ml tube (1.2 × 6 cm) sealed on perpendicularly to the side of the flask about 1 cm below the standard taper joint (Fig. 1). Samples of lipids 0.1 to 100 mg can easily be handled with this size flask. For larger samples, the apparatus may be scaled up proportionately; e.g., a 125 ml flask with a 10–15 ml side tube can be used for samples up to 5 g.

The flask is designed for use with any pair of immiscible solvents when repeated extraction of the lower phase by the upper phase is desired. An amount of the lower phase just sufficient to fill the side tube (approx. 5 ml) is added to the flask, followed by 5–10 ml of the upper phase. After shaking the mixture and allowing the phases to separate, the flask is slowly tilted to a horizontal position, so that the lower phase runs into the side tube, as shown in Fig. 1. At the same time, the upper phase will run out of the flask and is collected in a suitable receiver; removal of all the upper phase is not necessary. After rinsing the mouth of the flask with solvent, the flask is returned to the normal vertical position, more upper phase is added, and the process is repeated at least twice more, or as many times as is found necessary. Quantitative recovery of both the upper and the lower phases is readily achieved by this procedure.

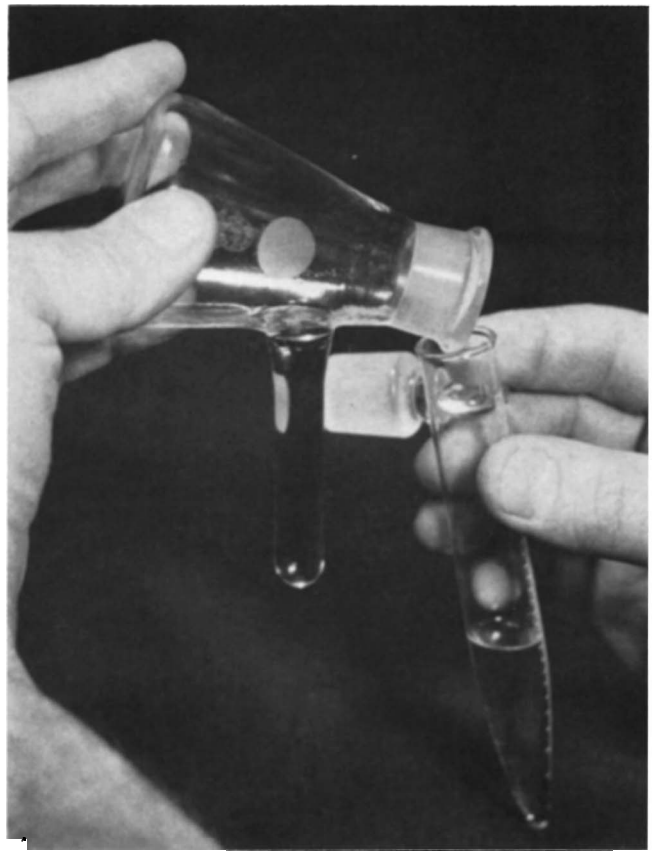

FIG. 1. Modified flask for cleavage of lipids and separation of products, in position for separation of immiscible phases.

The pairs of solvents found to be most useful were methanol–water 4:1 or 9:1 (v/v) and petroleum ether (bp 30–60°). With this system, emulsions are rarely encountered; and the petroleum ether extract, since it contains virtually no water, need not be treated with drying agents prior to evaporation of the solvent.

Typical Applications

(*a*) *Methanolysis of Lipids Containing O- or N-Acyl Groups.* The flask is particularly useful in preparing fatty acid methyl esters by the method of Stoffel, Chu, and Ahrens (2). The sample (50 mg or less), in 4.0 ml of methanolic hydrogen chloride solution (2.5%, ca. 0.7 N, made by passing hydrogen chloride gas from a cylinder into reagent grade methanol), is heated under reflux (calcium chloride tube) on a hot plate for 1–2 hr; 1 ml of water is added, the volume of methanolic phase is adjusted, if necessary, to fill the side tube by addition of methanol, and the fatty acid methyl esters are extracted with petroleum ether as described above. The combined petroleum ether extracts are evaporated to dryness in a stream of nitrogen (bath, 30°) and the methyl esters are weighed and analyzed directly by GLC. Recovery of methyl esters from free fatty acids, glycerides, galactosyl diglycerides (3), lecithin, and cephalin is quantitative (Table 1).

The methanolic phase may be made to a known volume and analyzed for the various water-soluble constituents; e.g., total P from phosphatides (Table 1), glycerol and glycerophosphate from glycerides and phosphatides (4), and glycerol and galactose from galactosyl diglycerides (3).

Methanolysis of sphingolipids by procedure (*a*) could not be tested with synthetic or natural samples of known fatty acid and long-chain base composition, since these were not available. However, some indication of the applicability of procedure (*a*) to sphingolipids was obtained using partially purified preparations of sphingomyelin and cerebrosides from ox brain (kindly supplied by Dr. N. Fisher, Baking Industries Research Assoc., Chorleywood, Herts, England).

The samples were refluxed with 2.5% methanolic hydrogen chloride 3.5–5 hr, and the fatty acid methyl esters extracted with petroleum ether as described in procedure (*a*). The methyl esters were weighed, analyzed for ester groups (5), and subjected to GLC on a short column (21 in.) of GE-silicone (SE-52) at 198° (6). Long-chain α-hydroxy acids could be analyzed on this column without prior conversion to methoxy or acetyl derivatives (cf Carroll [7]).

To obtain the long-chain bases, the methanolic phase was concentrated in the flask in a stream of nitrogen (bath, 35°) to a small volume, made alkaline with 0.5 ml of 7 N sodium hydroxide, the volume adjusted with water to fill the side tube, and the bases extracted with ethyl ether; the ethyl ether extracts were concentrated in vacuo to dryness and the residue was weighed and chromatographed on silicic acid-impregnated paper with diisobutyl ketone–acetic acid–water 40:25:5 (v/v) according to Marinetti et al. (8).

TABLE 1 RECOVERY OF FATTY ACID METHYL ESTERS AFTER METHANOLYSIS OF INDIVIDUAL LIPIDS

Lipid	Sample		Amount of Methyl Ester Recovered		
	mg	*μequiv.*	*mg*	*μmole**	*%*
Stearic acid	30.3	106.5	31.4	105.3 (104.5)	98.4
1,2-Distearin	33.6	107.5	32.0	107.2 (108.5)	100.3
Palmitoyl-1,2-distearin	31.7	110.1	32.6	112.8 (111.5)	101.7
Monogalactosyl-1,2-distearin†	15.1	38.4	11.6	38.9	101.2
Digalactosyl-1,2-distearin†	14.0	29.6	8.9	29.8	100.8
L-α-(Dimyristoyl)-lecithin‡	25.7	73.8	17.3	71.5	97.0
L-α-(Dipalmitoyl)-cephalin§	28.1	81.2	21.8	80.6	99.4

* Calculated from the weight of methyl ester; values in brackets are ester values as determined by the hydroxamic acid method (5).

† Prepared as described elsewhere (3).

‡ Synthetic product from LaMotte Chemicals Co. Estimation of P in the methanol phase gave 38.0 μmole (103% recovery).

§ Synthetic product (A grade) from California Corporation for Biochemical Research. Estimation of P in the methanol phase gave 40.2 μmole (99.0% recovery).

The sample of ox brain sphingomyelin (Anal. Found: C, 66.2; H, 11.7; N, 3.4; P, 3.8%; N/P atomic ratio, 1.96. Calcd. for lignoceroyl or nervonoyl sphingomyelin: C, 67.7–67.9; H, 11.7–11.5; N, 3.36–3.37; P, 3.74%. Calcd. for stearoyl sphingomyelin: C, 65.7; H, 11.4; N, 3.74; P, 4.15%) gave the following results after 5 hr methanolysis: 12.6 mg (15.5 mμ moles based on P determination) yielded 5.6 mg of methyl esters (16.5 μmoles [107%] based on average mol wt 340, as determined by GLC), consisting of 2% $C_{16:0}$, 44% $C_{18:0}$, 2% $C_{20:0}$, 4% $C_{20:1}$, 33% $C_{24:1}$, 7% HO-$C_{??:0}$, and 6% HO-$C_{24:0}$;[1] 3.6 mg of long-chain base (12.0 μmoles, 78%, assuming a C_{18} base), which gave only one spot (R_F, 0.52–0.54), staining yellow with Rhodamine and strongly positive with ninhydrin and periodate-Schiff reagents; 14.9 μmoles of the lipid phosphorus (96%) was recovered in the aqueous phase after extraction of methyl esters and long-chain base.

The sample of ox brain cerebrosides (Anal. Found: C, 69.7; H, 11.2; N, 1.87; P, 0.0. Calcd. for oxynervon:

[1] The GLC analysis shows that the sample probably still contains some cerebrosides and is mostly a mixture of the stearoyl and nervonoyl sphingomyelins; lignoceroyl sphingomyelin can only be present in traces and may have been removed during the preparation of this sample (cf analyses obtained by Rennkamp [9]).

TABLE 2 RECOVERY OF NONSAPONIFIABLE MATERIAL AND FATTY ACIDS

Degradation Procedure	Lipid Mixture	Amount of Sample		Nonsaponifiable Matter Recovered		Fatty Acid Recovered		
		mg	*μmole*	*mg*	*%*	*mg*	*μmole*	*%*
Methanolic HCl; NaOH	Palmitaldehyde dimethyl acetal*	17.7	...	16.9	95.5	16.8	73.5	100.8
	+ L-α-Dimyristoyl lecithin	25.4	36.5					
Methanolic HCl; NaOH	Cholesterol	19.5	...	19.6	100.5	16.7	73.1	101.0
	+ L-α-Dimyristoyl lecithin	25.2	36.2					
Methanolic NaOH	Cholesterol	20.4	...	20.7	101.2	17.6	77.2	98.5
	+ L-α-Dimyristoyl lecithin	27.3	39.2					

* Prepared from synthetic palmitaldehyde (15) by procedure (*b*) and purified by GLC.

C, 69.9; H, 11.1; N, 1.70) gave the following results after 3.5 hr methanolysis: 29.7 mg (36.0 μmoles, based on mol wt 826, calculated from results of GLC) yielded 15.5 mg of methyl esters (38.8 μ mole [107%], based on average mol wt 397, as determined by GLC; ester determination, 27.2 μmoles [76%]), consisting of 14% HO-$C_{18:0}$, 3% HO-$C_{22:0}$, 7% $C_{24:0}$; 50% HO-$C_{24:1}$, 16% HO-$C_{25:1}$, and 10% HO-$C_{26:1}$;[2] 8.0 mg of long-chain base (26.8 μmoles, 75%, assuming a C_{18} base), which had the same chromatographic properties as that from the sphingomyelin sample.

These results show that methanolysis of sphingolipids in 2.5% methanolic hydrogen chloride for periods up to 5 hr gives only a 75–78% cleavage of the fatty acid amide linkage but results in almost complete cleavage of phosphoryl choline from sphingomyelin and, presumably, of the sugar from cerebroside. The high recovery of methyl esters on a weight basis is probably due to the concomitant extraction of intact ceramide, as was also found by Kishimoto and Radin (10) even after methanolysis of cerebrosides in saturated methanolic hydrogen chloride for 16 hr. This deficiency must be considered, therefore, when natural lipid mixtures containing sphingolipids are treated by procedure (*a*).

A more complete methanolysis of the cerebroside sample was achieved using the conditions of Sweeley and Moscatelli (11), namely, refluxing with methanolic 2 N hydrochloric acid (methanol–conc. hydrochloric acid 5:1 [v/v]) for 5 hr.: 28.3 mg (34.2 μmoles) gave 14.1 mg of methyl esters (35.5 μmoles, 103% on weight basis; 32.0 μmoles, 94%, by ester determination), and 10.4 mg of long-chain base (34.8 μmoles, 101.5%). Therefore, this methanolysis procedure is recommended for samples containing sphingolipids.

(*b*) *Methanolysis of Lipid Mixtures Containing Acyl Esters, Plasmalogens, and Nonsaponifiables.* The sample (1–50 mg) is first treated with methanolic HCl (4.5 ml) as described under procedure (*a*). To the cooled methanolysate is added a drop of phenolphthalein and 0.5 ml of 7 N aqueous sodium hydroxide (sodium chloride crystals appear but do not interfere), and the mixture is refluxed for 1.5 hr. The aldehyde dimethyl acetals and nonsaponifiable material are then extracted with petroleum ether as described above; the extracts are evaporated to dryness under nitrogen; and the residue is weighed and analyzed for dimethyl acetals (12, 13), long-chain alcohols (14), and sterols (if present) by GLC.

The methanol phase is then acidified strongly with 0.4–0.5 ml of 6 N sulfuric acid (sodium sulfate precipitates but does not interfere), the volume is adjusted to fill the side tube using methanol, and the free fatty acids are extracted with several portions of petroleum ether. The extract is evaporated to dryness under nitrogen; and the fatty acids are weighed, converted to methyl esters with diazomethane in ether (or as described under procedure [*a*]), and analyzed by GLC.

Since pure samples of plasmalogen lecithin or cephalin were not available, procedure (*b*) was tested with a mixture of pure palmitaldehyde dimethyl acetal and synthetic dimyristoyl lecithin. Virtually complete separation and recovery of the acetal from myristic acid (derived from the dimyristoyl lecithin) was achieved (Table 2). Separation and recovery of cholesterol from myristic acid, after methanolysis of a mixture of cholesterol and dimyristoyl lecithin, were also quantitative (Table 2).

If the mixture does not contain plasmalogens, this procedure can be simplified by hydrolyzing the sample in 4.5 ml of methanol plus 0.5 ml of 3 N NaOH for 1.5 hr, extracting the nonsaponifiable material, then acidifying the methanol phase and extracting the fatty acids as described above. Recovery of the products in this variation is also quantitative (Table 2). Hydrolysis with methanolic sodium hydroxide could also be used with glycerolphosphatides and/or glycerides alone.

When separate analysis of fatty acids, aldehydes, and other nonsaponifiables is not desired, and for preliminary scanning of the hydrolysis products, the hydrolysis procedure may be further simplified by using procedure

[2] The GLC analysis shows that this sample is rich in oxynervon and contains not more than traces of cerebron; either the starting material contains low amounts of cerebron or this component was removed during preparation of the sample; identity of the HO-$C_{25:1}$ and HO-$C_{26:1}$ components is tentative.

(*a*) alone. In this case, the petroleum ether extract will contain methyl esters, aldehyde dimethyl acetals, and nonsaponifiables; and GLC analysis will show the relative proportions of aldehyde acetals (and nonsaponifiables such as long-chain alcohols and sterols) in relation to fatty acids.

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