Isolation of polar lipids from triglyceride mixtures*

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» Mixtures of phosphatides and cerebrosides (polar lipids) isolated from animal tissues by conventional extraction methods frequently contain an appreciable amount of neutral lipids (triglycerides). Methods frequently used in fractionating lipid mixtures (e.g., repeated acetone precipitations of the polar lipids) offer limited possibilities for a quantitative isolation of the phosphatides and cerebrosides occurring in a given source.

Removal of triglycerides from complex lipid mixtures was successfully achieved in 1952 by Borgström (1) using silicic acid chromatography. This chromatographic method has been very useful for fractionating mixtures containing polar as well as neutral lipids, but it involved an expensive and rather tedious technique when applied to gram quantities. Frankel *et al.*(2), applying silicic acid chromatography to isolate a small amount (43 mg) of phospholipids from a cream lipid fraction containing 20 g of triglycerides, eluted a 100-g column of silicic acid with 4,650 ml of organic solvents during 26 to 40 hours.

Data obtained in the early stages of our research program concerning milk lipids indicated that a quantitative separation of polar lipids from mixtures containing a large excess of triglycerides could actually be obtained using simple extraction techniques. Further studies in this direction led to a simple method successfully applied in the isolation of polar lipids from milk and egg yolk. Mixtures of polar lipids containing triglycerides in excess are submitted to a relatively small number of countercurrent distributions, using a binary system formed by mixing equal volumes of petroleum ether and 87% ethanol.[1]

EXPERIMENTAL METHODS

The solvent systems used were prepared from reagent grade absolute ethanol and petroleum ether (b.p. 40°–70°), not additionally purified. All other solvents were also of reagent grade.

Mixed Lipids from Milk. A fraction containing the total lipids in cow's milk was obtained as follows:

Fresh whole milk was evaporated *in vacuo* to dryness (flash evaporator) at 37°-40°. The gummy residue was triturated with acetone. The acetone was removed *in vacuo* at room temperature and discarded. The material thus obtained was extracted three times with chloroform-methanol mixtures. The extractions were carried out at room temperature (30 minutes, occasional shaking) using two volumes (v/w) of solvent, each time containing different chloroform-to-methanol ratios (2:1, 1:1, and 1:2 [v/v]). The combined extracts were freed from nonlipid contaminants according to the procedure devised by Folch *et al.* (7). The fraction thus obtained contained, as shown by conventional analytical methods, practically the total amount of the lipids present in cow's milk.

Mixed Lipids from Egg Yolk. A fraction containing the total lipids was prepared from fresh egg yolk using the procedure mentioned above.

Neutral Lipids from Milk. A pure fraction of the

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[1] Petroleum ether-ethanol (or methanol)-water systems have previously been used by several workers (3 to 6) in countercurrent distribution experiments attempting complete separation of particular lipids present in various mixtures of polar lipids.

neutral lipids present in cow's milk was isolated from commercial butter treated as described in the following section with the petroleum ether-87% ethanol system.

Sphingolipids from Beef Brain.[2] The crude sphingolipid fraction investigated was prepared from beef brain according to the method of Carter *et al.* (8).

Fractionation Scheme. The extracts of mixed lipids were evaporated *in vacuo* to dryness at room temperature. The residue thus obtained (10 g) was dissolved in 45 ml of the solvent system's upper phase; the system used being prepared by mixing equal volumes of petroleum ether and 87% ethanol. The solution thus formed was transferred to a separatory funnel and mixed thoroughly with 15 ml of the solvent system's ethanol phase. The equilibrated[3] lower phase of the system thus obtained was then transferred to a second separatory funnel containing 45 ml of the preequilibrated petroleum ether phase. Lower phase (15 ml) of the petroleum ether-87% ethanol system was added to the first separatory funnel, both funnels were shaken thoroughly, and the systems were allowed to equilibrate.[4] The lower phase of the second separatory funnel was then withdrawn to an Erlenmeyer flask, and the lower phase of the first funnel was transferred to the second one. The procedure mentioned above was repeated six times. The last portion of the solvent system's lower phase (15 ml) added to the first separatory funnel was completely withdrawn from the system, i.e., no lower phase of the petroleum ether-87% ethanol system was added to the first separatory funnel during the last stage of the experiment.

The combined extracts (8 x 15 ml) finally removed from the system contained practically the total amount of the polar lipids, contaminated with 0.02% to 0.03% of the neutral lipids present in the original fraction.

The fractionation scheme described in this section is actually a countercurrent distribution carried out by the single withdrawal procedure[5] devised by Craig *et al.* (9), in which the samples of the upper stationary phase remaining in the two separatory funnels compose the fundamental series,[6] and the eight samples of the lower mobile phase removed from the system form the withdrawn series.[7]

Recovery of the Lipids. The eight lipid extracts (ethanol layers) composing the withdrawn series were combined and the solution thus obtained was concentrated *in vacuo* at room temperature to approximately one-fifth of its original volume (25 ml). Lipids can quantitatively be extracted from this solution with chloroform by the method devised by Folch *et al.* (7); 40 ml of chloroform was used for the extraction mentioned above.

[2] Kindly donated to the authors by Professor H. E. Carter, University of Illinois, Urbana, Illinois, U.S.A.
[3] A clear separation of the phases takes place in 3 to 4 minutes.
[4] See footnote 3.
[5] Terminology according to (9).
[6] See footnote 5.
[7] See footnote 5.

TABLE 1. PARTITION RATIOS OF VARIOUS LIPIDS IN A NUMBER OF PETROLEUM ETHER-ETHANOL-WATER SYSTEMS*

Alcohol Concentration of the Ethanol Solution Used, %	Partition Ratios†				
	Neutral Lipids from Milk‡	Polar Lipids from Milk§	Polar Lipids from Egg Yolk§	Sphingomyelins from Beef Brain§	Cerebrosides from Beef Brain‖
92.0	0.115	1.26	1.90	—	—
91.0	—	1.62	1.99	3.50	4.10
90.5	0.060	—	2.15	3.70	4.70
90.0	—	2.41	2.80	4.50	5.02
87.5	0.040	5.30	5.34	5.55	6.82
86.0	0.033	4.38	3.64	5.40	7.40
83.0	0.026	2.73	1.93	4.80	6.25
81.5	0.018	2.40	1.70	4.30	5.45
77.0	—	—	1.61	4.05	—

* The systems used were prepared by mixing petroleum ether with an equal volume of an aqueous ethanol solution.
† The amount of lipid present in the lower phase was divided by the amount of lipid found in the upper phase.
‡ The total amount of solids present in the solutions investigated was determined.
§ Based on the amount of phosphorus present in the two phases.
‖ Based on the amount of carbohydrate present in the two phases. This was determined according to the method of Dubois *et al.* (10).

RESULTS

In a series of preliminary experiments, the partition ratios of several lipid samples (Table 1) in various petroleum ether-ethanol-water binary systems were experimentally determined. All binary systems investigated were prepared by mixing petroleum ether with an equal volume of an aqueous ethanol solution of varying alcohol concentration. It was shown that the polar lipids investigated have a maximum partition ratio (in favor of the polar phase) in petroleum ether-ethanol-water systems prepared with aqueous ethanol solutions containing alcohol in concentrations 86% to 88% (v/v), while a steady increase of the alcohol concentration of the ethanol solution used resulted in a constant increase of the triglyceride partition ratio.

It was furthermore observed that the phases of these petroleum ether-ethanol-water systems remain practically constant in volume for temperature changes

TABLE 2. FRACTIONATION OF TWO LIPID MIXTURES

Fraction	Total Solids (mg)	Total Phosphorus* (μg)	Recovery of Phosphorus (%)
Lipid mixture A†	2995	1050	—
Combined ethanol layers	34§	1046	99.7
Combined p. ether layers	2961	0	—
Lipid mixture B‡	5637	2250	—
Combined ethanol layers	74§	2272	101
Combined p. ether layers	5563	0	—

* According to the method of Sperry (11) as modified in (12).

† Lipid mixture A contained the total lipids present in whole milk.

‡ Lipid mixture B was an artificial mixture containing lipids from egg yolk and triglycerides from cow's milk.

§ By difference.

varying between 20°–30° only in cases of systems prepared with aqueous ethanol solutions of an alcohol concentration not higher than 90% to 91% (v/v). The experiments described in this paper were carried out at room temperature; i.e., 18°–22°.

Two mixtures of neutral and polar lipids were successfully fractionated, namely: (A) a mixture containing the total lipids present in whole milk, and (B) an artificial mixture containing lipids from egg yolk and triglycerides from cow's milk at a ratio of 1:100. In both cases the total amount (99.7% to 101%) of the polar lipids present in the mixtures was recovered from the withdrawn polar phase of the petroleum ether-87% ethanol system used (Table 2). Possible contamination of the polar lipid fraction with neutral lipid, while unlikely in view of the widely different partition ratios observed, has not been ruled out.

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