Distribution of Lipids in Various Fractions of Cow's Milk

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The distribution of lipids in various milk fractions (cream, skim milk, casein, whey, and separator slime) was studied. Petroleum ether and chloroform–methanol (2 to 1) were used successively as solvents. The petroleum ether fraction is referred to as "free" lipid and the chloroform–methanol fraction as "bound" lipid. Neutral lipids were found in both the free and the bound fractions in milk. Phospholipids were found in the bound fraction only. The composition of the glycerides was determined by thin layer chromatography. The diglyceride and monoglyceride content of the bound neutral lipid fraction was much higher than that of the free neutral lipid fraction. No significant difference in the composition of the phospholipids was observed among the various milk fractions.

Previous studies found that the lipids associated with acid-precipitated casein are not completely extractable by only one solvent (Cerbulis and Zittle, 1965). Even the chloroform-methanol (2 to 1) mixture did not remove all casein lipids, and some were removed only by saponification (Cerbulis and Zittle, 1965).

The present studies determined the amount of "free" and "bound" lipids in all major milk fractions under conditions more nearly approaching their natural state in milk. The nature of the lipid fractions was determined by thin layer chromatography (TLC). The "free" lipids of milk fractions were extracted from the freeze-dried milk solids with petroleum ether and the "bound" lipids released by a further extraction with chloroform–methanol (2 to 1, v./v.). This arbitrary classification of lipids as "free" and "bound" has been accepted by many lipid chemists.

EXPERIMENTAL

Solvents and Reagents. All solvents were reagent grade and freshly redistilled. Iodine vapor was used for general staining, ninhydrin (Levy and Chung, 1953) for proteins, and *p*-anisidine (Cerbulis, 1955) for carbohydrates.

Preparation of Milk Fractions. Milk (39 liters) of Holstein cows was separated as shown in Figure 1. Cream, crude casein, and separator slime were freeze-dried without additional treatment. Skim milk and whey were dialyzed against distilled water for 2 days using cellulose dialysis tubing, then concentrated to a small volume in vacuo and finally freeze-dried.

Lipid Extraction. The free lipids were extracted from the freeze-dried samples with petroleum ether (1 liter per 100 grams of solids) by continuous stirring for 3 hours and the extract was removed by filtration. The extraction was repeated until the extract contained no lipids. The extract was evaporated to dryness on a steam bath with a stream of nitrogen and the residue weighed. Usually, five to 12 extractions were performed. The residue of each extract was analyzed by TLC. Composition of the combined lipid residue was determined by TLC and column chromatography.

The bound lipids were next extracted from these samples with chloroform–methanol (2 to 1, v./v.), as described by Folch *et al.* (1957), by continuous stirring for 3 to 4 hours using 20 ml. of solvent per gram of dry milk solids. The samples were extracted three times. The extracts were evaporated in vacuo to dryness, then the residue was taken up with a small volume of chloroform-methanol (2 to 1) and washed twice with 0.2 volume of water, as described by Folch *et al.* (1957), to remove nonlipid contaminants. If an insoluble solid was formed in the water washing procedure, it was removed with the water phase. The chloroform layer (lipids) was evaporated to dryness in vacuo, and the lipid residue was analyzed by TLC and column chromatography. The wash water was dialyzed, and the nondialyzable fraction was evaporated to dryness and the residue was saved for further studies.

The author observed that the chloroform–methanol (2 to 1) extract, after removal of solvent, was not completely soluble again in the same solvent because of physical changes in the dry residue.

Separation of Neutral Lipids from Phospholipids. The crude lipid mixture was separated by the silicic acid method described by Abramson and Blecher (1965). The recovery of lipids was quantitative. TLC showed that the separation was complete.

Thin Layer Chromatography (TLC). The technique was similar to that described by Stahl (1958) and Rouser *et al.* (1963). Silica gel G, as well as the thin layer equipment, was purchased from Brinkmann Instruments, Great Neck, L.I., N.Y. The plates were

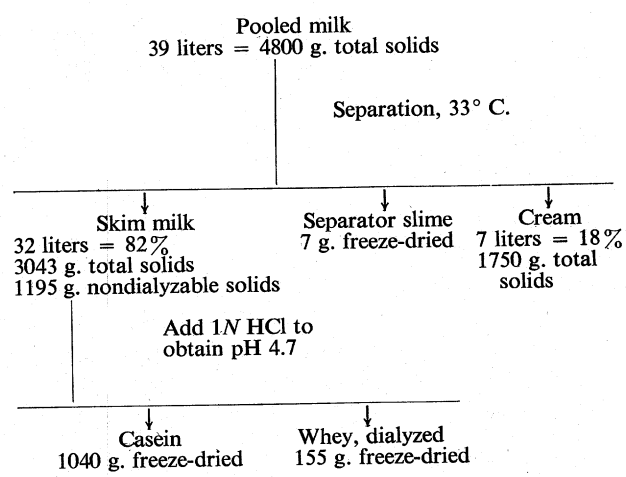

Figure 1. Fractionation of milk

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heated for 1 to 2 hours at 110° to 115° C. prior to use. The developing solvents were petroleum ether–diethyl ether–acetic acid (90:10:1, v./v./v.) for neutral lipids and chloroform–methanol–water (65:25:4, v./v./v.), for phospholipids. Iodine vapor was used to make the lipids visible.

Preparative TLC. The sample, dissolved in benzene, was applied along the base line of the plate (20 × 20 cm.) as a streak in amounts up to 20 mg. After the development of the plate, the solvent was allowed to evaporate from it. Then the plate was covered with another smaller plate (15 × 20 cm.), and the strip not covered was treated with iodine vapor which delineated the components. The corresponding unstained streaks were scraped off the plate with a spatula and delivered to a large test tube. For neutral lipids, 15 ml. of diethyl ether were added. The mixture was stirred with a glass rod for 5 to 10 minutes and centrifuged, and the extract was evaporated in a weighed beaker with the aid of a stream of nitrogen. The extraction was repeated three times. The lipids were dried at 105° C. to constant weight. For the extraction of phospholipids from the adsorbent, chloroform–methanol–water–formic acid (65:25:4:2, v./v./v./v.), was used and the procedure followed as described for neutral lipids.

Column Chromatography. Mono-, di-, and triglycerides were determined by chromatography on a column packed with silica gel (Davison Chemical Co., Baltimore, Md., grade 923, 100- to 200-mesh), as described by Distler and Baur (1965). The results obtained by this procedure were comparable to those obtained by preparative TLC (Table I).

RESULTS AND DISCUSSION

The results are reported in Tables I and II and Figures 2 and 3.

Aqueous Washing. The nondialyzable aqueous layer of the original chloroform–methanol (2 to 1, v./v.) extracts gave a positive ninhydrin test and all of the material remained at the origin or migrated more slowly than lecithin when this fraction was examined by TLC. The residue of this fraction was a white solid, and a part of this was readily soluble in 50% aqueous methanol. The residue contained 12.49% N and 0.29% P.

Lipid Distribution in Milk Fractions. Table I shows the lipid distribution in freeze-dried milk fractions. Separator slime contained almost as much of lipids as cream but the bound lipid content was considerably higher. The proteins of whey contained 0.8% of lipids, and 84.6% of them were in bound form. Also, 43.4% of the casein lipids were in the bound form.

In the present investigation, chloroform–methanol (2 to 1) extracted casein was extracted again with chloroform–methanol (7 to 1, v./v.) saturated with concentrated aqueous ammonia (about 5.5%, v./v.) as suggested by Rouser *et al.* (1963). The extraction gave 0.12% of additional lipids (neutral fats and phospholipids). This last extraction

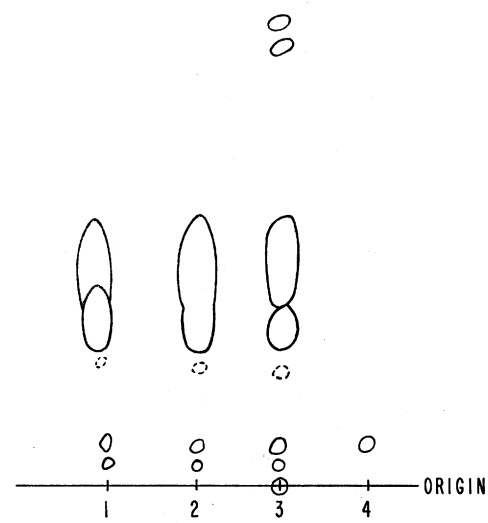

Figure 2. Thin layer chromatography for neutral lipids

Solvent. Petroleum ether–diethyl ether–acetic acid (90:10:1, v./v./v.)

1. Free lipids
2. Butter oil
3. Bound lipids
4. Cholesterol

Table I. Components in Grams per 100 Grams of Freeze-Dried Solids

	Cream		Skim Milk[a]		Casein		Whey[a]		Separator Slime	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Neutral lipids	79.10	0.03	1.15	0.36	0.82	0.55	0.12	0.53	72.90	0.84
Phospholipids	...	0.27	...	0.29	...	0.15	...	0.18	...	2.26
Total lipids	79.10	0.30	1.15	0.65	0.82	0.70	0.12	0.71	72.90	3.10
Proteins	20.60[b]		98.20		98.48		99.17		24.00	

[a] Nondialyzable fraction.
[b] Total nonlipid solids.

Table II. Glyceride Composition of Neutral Lipids, Per Cent

Components	Cream		Skim Milk		Casein		Whey		Slime	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Triglycerides	96.4	10.0	80.9	65.5	86.3	96.2	58.1	62.9	90.8	94.2
Diglycerides	3.3	80.0	18.2	32.8	13.1	2.7	25.7	28.8	6.1	3.8
Monoglycerides	0.3	10.0	0.9	1.7	0.6	1.1	16.2	8.3	3.1	2.0

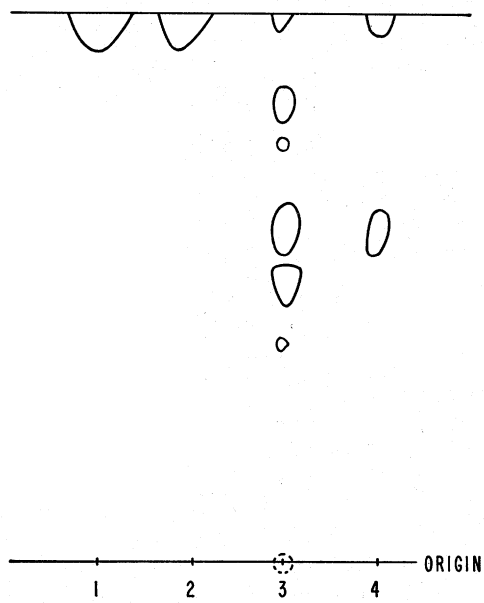

Figure 3. Thin layer chromatography for phospholipids

Solvent. Chloroform–methanol–water (65:25:4, v./v./v.)

1. Butter oil + cholesterol
2. Free lipid fraction
3. Bound lipid fraction
4. Lecithin + butter oil

showed that a small amount of lipids was bound very tightly to the proteins and was not extractable by common lipid solvents. The free lipid fraction of casein and whey protein had a higher content of diglycerides and monoglycerides, both more polar than triglycerides, than the corresponding lipid fraction of cream (Table II).

Total Lipids. The phospholipid contents of skim milk, casein, and whey lipids were high (16.2, 9.6, and 21%, respectively, of the total lipids were phospholipids) in comparison with cream, in which the phospholipid content amounted to 0.3% of the total. Rewald (1937) found that 0.24% of dry whole milk consisted of phospholipids.

Bound Lipid Fraction. Phospholipids are associated with proteins in milk and their content in bound lipid fractions is relatively high. Cream-bound lipids contained 90% phospholipids, skim milk 44%, casein 21.9%, whey 25.5%, and separator slime 72.7%. Bird *et al.* (1935) have reported that the lipid fraction of buttermilk contained 21.2% phospholipids, 2.9% sterols, and 75.9% neutral lipids.

All the phospholipid fractions contained cephalin, lecithin, and sphingomyelin. These components were identified by TLC and were in all milk fractions. No significant differences were observed among the various milk fractions. Some minor components, not identified, were also observed.

Free Lipid Fraction. The petroleum ether–extractable free lipid fractions did not contain phospholipids (or less than 0.01%) when these fractions were examined by TLC, or by silica gel column chromatography (Distler and Baur, 1965), and by the batchwise silicic acid method (Abramson and Blecher, 1965). This showed that the phospholipids were not free in milk, but were associated with the proteins and perhaps with inorganic cations (Rojas and Tobias, 1965) or other milk constituents. The composition of glycerides of the free lipids is shown in Table II. The free lipids of cream, casein, and slime contained only a trace amount of an unknown colorless material, probably hydrocarbons. The free lipids of whey contained up to 56% of an uncommon component in the sterol ester–hydrocarbon region on the TLC plate. This component was not investigated further. The free lipid fractions of casein and whey contained more diglycerides and monoglycerides in comparison with the free lipid fraction of cream.

Neutral Lipids. The glyceride composition of the free and bound lipid fractions was studied (Table II). Diglycerides and monoglycerides, as polar lipids, show affinity to proteins, and are concentrated in the lipid fractions of casein and whey and the bound lipid fraction of cream. The neutral lipids of the bound lipid fraction of cream contained up to 90% of di- and monoglycerides.

Lipid-Protein Complexes of Milk. A part of the neutral lipids and all of the phospholipids of cow's milk are bound lipids in the sense that they are not extracted by petroleum ether, but they are extracted by more polar solvents such as chloroform–methanol. The nature of this binding cannot be stated precisely but may be due to association between lipid and protein, perhaps in definite lipid-protein complexes. If the amount of lipids obtained by extraction of casein with chloroform–methanol–ammonia (0.12%) and by saponification (Cerbulis and Zittle, 1965) (0.3%) is added to the reported lipid content, then the total lipid content of milk lipid–protein complexes is higher than shown in Table I obtained with chloroform–methanol (2 to 1).

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