

State of Unesterified Fatty Acids in Skim Milk

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ABSTRACT

By a multiple extraction procedure the unesterified fatty acids of skim milk have been shown in at least three entities: a) free dissociated and undissociated fatty acids, b) fatty acids associated with the membrane material in skim milk, and c) fatty acids of unknown origin requiring acidification to pH 1.5 for extraction.

INTRODUCTION

In (7) we demonstrated that the dialyzable 6 through 12-carbon free fatty acids exist in equilibrium between the aqueous and non-aqueous fractions of skim milk. On the basis of the Henderson-Hasselbach equation we concluded that the fatty acids in the aqueous phase exist in skim milk for all practical purposes as the dissociated acids. This study is a continuation of our investigations into the phase distribution of the unesterified fatty acids of skim milk and reports our observations on the state of the acids in the nonaqueous phase.

MATERIALS AND METHODS

Lipid Extraction Sequence A

Water-ether Extract. Sixty milliliters of fresh pasteurized skim milk were lyophilized with a Virtis Freeze Drying Unit² (Virtis Research Equipment, Gardiner, NY). The resulting powder was ground thoroughly into 15 g of previously washed (4) and vacuum dried (65 C and .064 Atm for 16 h) silicic acid to give a free flowing powder. Six milliliters of distilled water were ground thoroughly into the powder in 1-ml increments, and the hydrated mixture (pH 6.1) was extracted 6 h (8 solvent turnovers

per h) in a Soxhlet Extractor with 200 ml of peroxide-free ethyl ether.

Acid-ether Extract. The silicic acid-skim milk powder from water-ether extract was removed from the thimble, the ether was evaporated by air drying for 1 h, and the hydrated powder was redried in a vacuum oven at 45 C and .064 Atm for 16 h. Five milliliters of 2.75 N H₂SO₄ were ground into the redried powder in 1-ml increments, and the acidified powder (pH 1.5) was extracted in a Soxhlet Extractor 6 h with 200 ml of ethyl ether.

Chloroform-methanol Extract. Following the acid-ether extraction the silicic acid skim milk mixture was recovered from the thimble, and the ether was removed by air drying. Two hundred milliliters of chloroform-methanol (2:1) were added to the powder, and the mixture was stirred magnetically for 3 h. The solvent was recovered by filtration, and the powder was washed with an additional 100 ml of chloroform-methanol. The combined filtrates were washed successively with two 75-ml volumes of .9% aqueous potassium chloride, and 75 ml of distilled water. The chloroform layer was dried with sodium sulfate, evaporated to dryness in a rotary evaporator under vacuum, and the residue was taken up in 20 ml of ethyl ether.

Lipid Extraction Sequence B

Water-Ether Extract. Same as described in A.

Chloroform-Methanol Extract. The lyophilized skim milk silicic acid mixture was redried as in A and extracted with 200 ml of chloroform-methanol (2:1) as described in A.

Acid-Ether Extract. The skim milk silicic acid mixture was recovered from the filter paper, partially dried in air, and the solvent was removed completely in a vacuum oven at 45 C and .064 Atm in 16 h. Five milliliters of 2.75 N H₂SO₄ were ground into the redried powder, and the mixture was extracted with 200 ml of ethyl ether as in A.

Acid-Chloroform-Methanol Extract. The silicic-acid lyophilized skim milk mixture was

Received October 14, 1978.

¹Agricultural Research, Science and Education Administration, US Department of Agriculture.

²Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

removed from the extraction thimble, the ether was removed under vacuum at room temperature, and the mixture was reextracted with 200 ml of chloroform-methanol as described in A.

Isolation and Methylation of Unesterified Fatty Acids

The fatty acids were isolated from the ether solutions according to the method of McCarthy and Duthie (4), and the methyl esters were prepared with 1% H₂SO₄ in anhydrous methanol as in (6).

Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) was with the Perkin-Elmer Model 900 Gas Chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with a flame ionization detector. The methyl esters were separated on a 1.83 m × .64 cm stainless steel column packed with 10% EGSS-X on 100/200 mesh Gas Chrom P. The flash heater and manifold were maintained at 230 C. The column was held at 90 C for 6 min following injection of the sample and immediately heated to 190 C (approximately 30-s programming time).

Unesterified Fatty Acid Quantitation

The methods limited quantitation of the fatty acids to the C₁₀ through C₁₈ fatty acids. Gas liquid chromatography calibration curves calculated by least squares were prepared for the methyl esters by chromatographing seven known amounts of each methyl ester and determining detector response by measuring peak heights. Concentrations of fatty acids were determined by reference to the calibration curves and the appropriate calculations.

Terminology

Dialysate — aqueous phase of skim milk obtained by vacuum dialysis (7, 8).

Dialyzable fatty acids — total unesterified fatty acids obtainable by repeated vacuum dialysis of skim milk according to (7).

Dialyzed fatty acids — unesterified fatty acids in the dialysate (aqueous phase) of skim milk.

Nondialyzed fatty acids — dialyzable unesterified fatty acids in the nonaqueous phase of skim milk.

Free fatty acids — nonesterified fatty acids

unassociated in aqueous and residual lipid phases of skim milk.

RESULTS AND DISCUSSION

Our initial studies comparing various procedures for extracting lipids from biological systems revealed quantitative as well as qualitative differences in the extractable unesterified fatty acids of lyophilized skim milk. It occurred to us, on the basis of these and other observations, that by employing several extraction procedures in the proper sequence, further insights into the state of the unesterified fatty acids of skim milk were possible.

Kintner and Day (2) were unsuccessful in isolating the unesterified fatty acids from a mixture of silicic acid and lyophilized milk by direct extraction with ethyl ether. They concluded that acidification of the dry mixture to pH 1.3 was required for complete extraction because "at the normal pH of milk, most of the acids in the aqueous phase should exist as salts", presumably not extractable as such with ether.

We also experienced difficulty in extracting unesterified acids from a mixture of lyophilized skim milk and silicic acid. However, fatty acids were extractable without acidification, providing the mixture was hydrated sufficiently (25 to 30% moisture). Furthermore, the relative concentrations of the acids extracted in this manner differed from the fatty acids subsequently extracted with ether following acidification to pH 1.5 of the redried water-ether extracted sample (Extraction Sequence A). The results obtained by extracting numerous samples of fresh skim milk showed that, of the total water-ether and acid-ether extractable fatty acids, a greater percentage of the lower molecular weight acids are extractable with the water-ether extraction system. The percentages of the individual acids, unlike those reported (7) for the dialyzable fatty acids in equilibrium between the nonaqueous and aqueous phases of skim milk, are not constant; they vary from sample to sample, especially for the higher molecular weight acids. In general, a greater percentage of the acid is extractable with the water-ether system as the total water-ether and acid-ether extractable fatty acid content increases in the sample. This observation is demonstrated in Table 1 which compares the fatty acids extracted from skim milks obtained

TABLE 1. Fatty acids C₁₀ through C₁₈ content of ethyl ether extracts of fresh and rancid skim milks extracted successively at pH 6.1 and 1.5.

FFA	Concentration ($\mu\text{g}/60$ ml of skim milk)							
	Fresh				Rancid			
	pH 6.1	pH 1.5	Total	pH 6.1	pH 6.1	pH 1.5	Total	pH 6.1
				—(%)—				—(%)—
C _{10:0}	25.6	3.7	29.3	87.4	362.6	45.0	407.6	89.0
C _{12:0}	35.3	11.7	47.0	75.1	369.2	71.6	440.8	83.8
C _{14:0}	80.4	46.5	126.9	63.4	892.4	234.0	1126.4	79.2
C _{16:0}	192.0	160.2	352.2	54.5	2762.1	686.8	3448.9	80.1
C _{18:0}	37.8	78.0	115.8	32.6	760.0	282.8	1042.8	72.9
C _{18:1}	83.4	203.6	287.0	29.1	1168.4	700.7	1869.1	62.5
C _{18:2}	7.8	43.7	51.5	15.1	127.4	115.4	242.8	52.5
Total	462.3	547.4	1009.7	45.8	6442.1	2136.3	8578.4	75.1

by centrifugation of fresh and laboratory produced rancid milk. These initial results suggested significant differences between the chemical states of the water-ether and acid-ether extractable fatty acids of skim milk.

A report (3) suggested that membrane lipids are not extracted by the usual silicic acid-sulfuric acid techniques; their extraction required multiple solvent systems including alcohol. Therefore, it was expected that a chloroform-methanol (2:1) extraction of a mixture of lyophilized skim

milk and silicic acid which had been extracted previously with water-ether and acid-ether (Extraction Sequence A) resulted in the further extraction of relatively large quantities of fatty acids, presumably those acids associated with the membranes of skim milk (Table 2). Significantly reversing the acid-ether and chloroform-methanol extraction sequence revealed that, whereas the acid-ether system (Extraction Sequence A) did not extract the membrane acids, the chloroform-methanol (Extraction Sequence B) did not extract the acid-ether extractable fatty acids. On this basis, we concluded that the acid-ether extractable fatty acids exist in skim milk in a different chemical state than the acids associated with the membrane material of skim milk. Further complicating the state of the fatty acids in skim milk was our final observation that complete recovery of the acid-liberated fatty acids in Extraction Sequence B required an additional chloroform-methanol extraction. The fatty acids extracted from a typical sample of fresh skim milk by the various solvent systems with Extraction Sequence B are shown in Table 3.

The unesterified fatty acids exist in skim milk in a variety of states distinguishable by extracting a mixture of the lyophilized sample and silicic acid with various solvent systems in the proper sequence: a) water-ether extractable fatty acids; b) chloroform-methanol extractable fatty acids, representing those acids readily

TABLE 2. Fatty acids extracted from a typical sample of lyophilized skim milk-silicic acid by various solvent systems by Extraction Sequence A.

FFA	Concentration ($\mu\text{g}/60$ ml of skim milk)			
	H ₂ O ether	Acid ether	Acid CHCl ₃ MeOH	Total
C _{10:0}	58.8	7.7	18.6	85.1
C _{12:0}	67.7	16.7	36.7	121.1
C _{14:0}	133.3	66.6	149.3	349.2
C _{16:0}	256.2	206.7	529.5	992.4
C _{18:0}	72.2	120.3	357.1	549.6
C _{18:1}	128.9	252.0	567.3	948.2
C _{18:2}	20.2	67.2	117.2	204.6
Total	737.3	737.2	1775.7	3250.2

TABLE 3. Fatty acids extracted from a typical sample of lyophilized skim milk-silicic acid by various solvent systems by Extraction Sequence B.

FFA	Concentration ($\mu\text{g}/60$ ml of skim milk)				Total
	H ₂ O ether	CHCl ₃ MeOH	Acid ether	Acid CHCl ₃ MeOH	
C _{10:0}	33.7	26.6	3.7	3.3	67.3
C _{12:0}	45.1	55.6	15.5	10.9	127.1
C _{14:0}	95.8	219.7	39.1	29.4	384.0
C _{16:0}	186.7	672.8	139.0	127.7	1126.2
C _{18:0}	58.8	276.6	118.3	120.4	574.1
C _{18:1}	106.7	408.8	214.8	177.8	908.1
C _{18:2}	13.5	44.9	58.1	39.2	155.7
Total	540.3	1705.0	588.5	508.7	3342.5

extracted from the membrane material of skim milk; and c) those acids requiring acidification to pH 1.5 prior to extraction which are divided further into ether extractable and chloroform-methanol extractable fatty acids. Although data in Tables 2 and 3 indicate that the nonesterified acids exist in distinct entities, further studies have suggested a possible relationship between the acid-ether and chloroform-methanol extractable fatty acids. In skim milks containing relatively high concentrations of acids, a portion of the acid-ether extractable fatty acids obtained in Extraction Sequence A is extracted

TABLE 4. Effect of pH on concentration of fatty acids extracted from lyophilized skim milk-silicic acid mixture with ethyl ether.

FFA	Concentration ($\mu\text{g}/60$ ml skim milk)			
	pH 6.1	pH 4.7	pH 3.0	pH 1.5
C _{10:0}	29.6	37.4	32.8	40.1
C _{12:0}	33.2	39.7	37.1	45.6
C _{14:0}	65.4	78.6	71.5	94.5
C _{16:0}	108.1	121.9	118.8	196.5
C _{18:0}	16.9	16.9	20.8	55.4
C _{18:1}	74.2	80.1	89.9	191.7
C _{18:2}	9.8	10.7	9.8	34.7
Total	337.2	385.3	380.7	658.5

by chloroform-methanol in Extraction Sequence B. Additional data obtained by these procedures will be necessary to verify such a relationship.

The water-ether extractable fatty acids appear to represent the dissociated and undissociated free fatty acids of skim milk. The partitioning of fatty acids between buffered solutions and organic phases depends on the relative solubilities of the acid in the phases, volume of the phases, and pH of the buffered solution. We observed that the C₁₀ and C₁₂ unesterified fatty acids in skim milk dialysate partitioned completely to ethyl ether when the dialysate (pH 6.7) was extracted as in (7). In contrast, approximately 95%, 80%, and 35% of the C₈, C₆, and C₄ acids were extracted by this procedure. Since ether extraction of the hydrated lyophilized skim milk-silicic acid mixture removed only trace amounts of the added water, the extraction procedure acted in effect as a water-ether partition system. Hence, it is reasonable that near quantitative extraction of the free dissociated and undissociated C₁₀ and higher molecular weight fatty acids from the hydrated mixture is achieved by Soxhlet extraction. The water-ether system was 90 to 95% effective in extracting the C₁₁ through C₁₇ odd carbon numbered fatty acids added to skim milk as the sodium salts prior to lyophilization. For a pK_a of 4.8 for the fatty acids of skim milk, acidification to pH 4.8 would result in a salt to acid ratio of 1:1, in contrast to a ratio of approximately 60:1 at the normal pH of skim

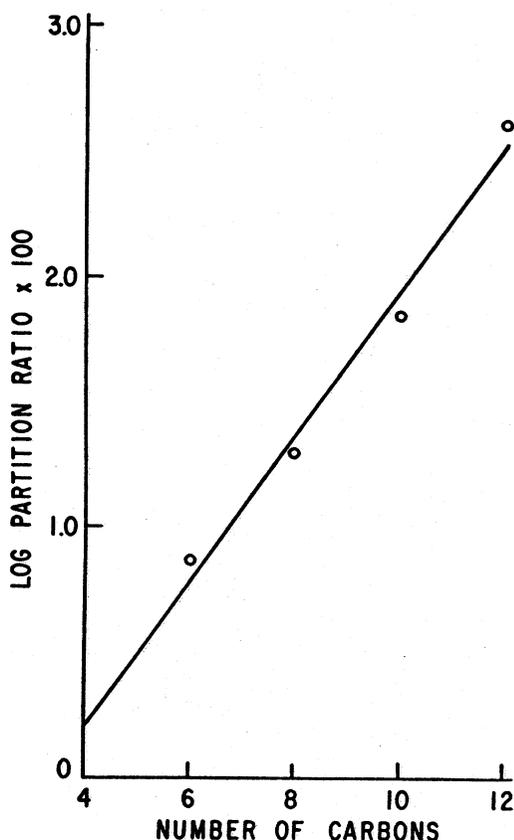


Figure 1. Effect of chain length on partition ratios of dialyzable fatty acids between nonaqueous and aqueous phases of skim milk.

milk. We demonstrated earlier (7) that as the pH of skim milk is decreased, the fatty acid content of skim milk dialysate also decreases, indicating a partitioning of the protonated fatty acids to the residual lipid phase of skim milk. In contrast, acidification of the lyophilized skim milk-silicic acid mixture (Table 4) did not result in significant increases in the extractable fatty acids until a pH of 1.5 was attained. The fatty acids requiring acidification to pH 1.5 for extraction do not represent the free fatty acids in the dissociated state; they are in skim milk in a state entirely different from the water-ether extractable acids. Therefore, the water-ether extractable acids include both the undissociated and dissociated free fatty acids of skim milk.

From data here and in the previous investigation (7), one can speculate on the ratio

of undissociated to dissociated free fatty acids in fresh skim milk. We demonstrated that the dialyzable C_6 through C_{12} free fatty acids exist in equilibrium between the nonaqueous and aqueous fractions of fresh skim milk in relatively constant ratios. We further demonstrated that as the pH of skim milk is decreased, the ratio of the nondialyzed to dialyzed C_6 through C_{12} dialyzable fatty acids increases, the reverse being true as the pH is increased. Those results, along with the solubility studies in skim milk dialysate, strongly suggest that the observed equilibrium of the fatty acids in skim milk is in reality a partitioning of the fatty acids between the residual lipid and aqueous phase of skim milk. Accordingly, the partition ratios of the individual dialyzable C_6 through C_{12} fatty acids between the nonaqueous and aqueous phases of skim milk represent, for all practical purposes, the ratios of the undissociated to dissociated state of the free fatty acids.

Figure 1 shows a plot of the log of the partition ratios (nondialyzed/dialyzed) times 100 vs. fatty acid carbon number based on the average data in (7). Least squares analysis ($\log P.R. \times 100 = .289N - .949$) ($r = .99$) showed that the partition ratios of the dialyzable fatty acids increased 3.8-fold with an increase in chain length of two methylene groups. As previously reported (7), the C_{14} and higher fatty acids have limited solubility in skim milk dialysate, and their partition ratios between the nonaqueous and aqueous fractions of skim milk are difficult to determine experimentally. However, by extrapolation of data in Figure 1, the partition ratios of the C_{14} , C_{16} , and C_{18} saturated acids are 12.5, 47.3, and 159.3, respectively.

The state of the acid-liberated fatty acids remains to be determined. Kintner and Day (2) reported that glyceride hydrolysis was avoided in an acid-ether extraction procedure when acidification was maintained below 1 ml 5.5 N H_2SO_4 /10 to 11 g of fluid milk. Unless skim milk contains readily hydrolyzed lipids of unknown structure, it is questionable whether the acid-liberated fatty acids are the result of glyceride hydrolysis since 1 ml of 2.75 N H_2SO_4 /12 ml of fluid skim milk was in the acid-ether extractions. The composition of the acid-liberated fatty acids, characterized by high C_{18} fatty acid content, resembles the C_{12} through C_{18} fatty acid content of commercial

bovine serum albumin (1) and the fatty acids extracted from egg yolk protein following proteolytic enzyme hydrolysis (5).

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