

## LIPIDS ASSOCIATED WITH ACID-PRECIPITATED CASEIN

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## ABSTRACT

Acid-precipitated casein contains 4.5-7% of lipids (on dry weight basis). About 40-50% of the total lipid is extractable with petroleum ether and is presumably free fat. The remaining 50-60% of the lipids are more strongly associated with the casein. Phospholipids appear mainly in the latter fraction; ratio of phospholipid to total lipids is greater in casein than in whole milk lipids.

Casein for laboratory use is usually prepared by repeated reprecipitation at the isoelectric point, washing with water and finally with fat solvents (alcohol, acetone, ether) (7). Part of the  $\beta$ - and  $\gamma$ -caseins are removed by these washings (4). Little is known about the lipid associated with acid-precipitated casein. The determination of the lipid content of casein has shown that different detection methods provide variable results (1). The lipid content of casein is also dependent on the method used for the preparation of casein (3). The present studies determined the amount of extractable lipids in acid-precipitated casein using fat solvents under conditions expected to remove total lipids, both free and bound. The nature of some of the fractions has been determined by thin-layer chromatography.

## EXPERIMENTAL PROCEDURE

*Preparation of isoelectric casein.* Casein was precipitated from fresh unpasteurized pooled skim milk at pH 4.7 by dropwise addition of 1 N HCl during continuous stirring. Casein was recovered by filtration. Various casein preparations were obtained from this as follows:

A. Standard casein was peptized twice at pH 7 by dropwise addition of 1 N NaOH, reprecipitated with 0.1 N HCl at pH 4.6, and washed four times after each precipitation with water at pH 4.7 (7).

B. Nonpeptized casein was washed five times with water at pH 4.6. Approximately 80% of the weight of both A and B preparations was water.

C. Freeze-dried casein was prepared without washing by the freeze-drying technique.

Lipid extractions were carried out with these caseins at 20 to 25 C. Samples of 200 g of casein (on dry weight basis) were extracted five times

with either petroleum ether or acetone (3.8 liters each extraction) by continuous stirring for 2-3 hr either separately or in sequence; two extractions by 4.5 liters each time with  $\text{CHCl}_3$  —  $\text{CH}_3\text{OH}$  (2:1). In some instances the treatment was concluded with extraction by 4.5 liters of  $\text{CHCl}_3$  —  $\text{CH}_3\text{OH}$  containing HCl (200:100:1, v/v/v) for 1 hr. The mixtures were filtered and the extracts were evaporated in vacuo to dryness by a rapid Craig vacuum technique.

*Saponification with NaOH.* After evaporation of the adhering fat solvent from the casein, the casein was saponified in two liters of 2.4 N NaOH for 2 hr at 90 C. After the reaction mixture was cooled and extracted with ether twice (unsaponifiable fraction), the mixture was acidified with concentrated HCl and extracted with ether twice (fatty acid). The extracts were washed with water, retained water removed with  $\text{Na}_2\text{SO}_4$ , and evaporated to dryness in vacuo.

Thin-layer chromatographic technique was similar to that described by Rouser et al. (9).

## RESULTS AND DISCUSSION

Results in Table 1 show that acetone removes 90-95% of lipids from the standard moist casein (A) and nonpeptized casein (B); the rest (5-10%) is extractable by  $\text{CHCl}_3$  —  $\text{CH}_3\text{OH}$  (2:1). Thin-layer chromatography shows that the acetone extracts contain neutral fats and phospholipids. The  $\text{CHCl}_3$  —  $\text{CH}_3\text{OH}$  extracts contain 2-5% neutral fat and phospholipids and 95-98% substances nonmigrating on thin-layer chromatography plates. The nonmigrative substances give positive reactions for fats and proteins and may be lipoproteins or lipopeptides (2). A comparison of the results obtained with Caseins A and B shows that precipitation and reprecipitation of the casein leads to the removal of lipids (about 20%).

About 37-47% of the total lipid of the freeze-dried casein is extractable with petroleum ether (C-a,b). This, presumably, is a measure of the free lipid associated with acid-precipitated case-

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TABLE 1  
Lipids in acid-precipitated casein, per cent, on dry weight basis <sup>a</sup>

Casein		Acetone	Petroleum ether (30-60 degrees b.p.)	CHCl <sub>3</sub> -CH <sub>3</sub> OH (2:1)	Total lipid
A. Standard Casein	(a)	5.22	—	0.26	5.48
	(b)	5.22	—	0.33	5.58
C. Freeze-dried Casein	(a)	6.72	—	0.35	7.07
	(b)	5.90	—	0.85	6.75
	(c)	—	3.40	3.90	7.30
	(d)	—	2.25	3.83	6.08
B. Nonpeptized Casein	(a)	2.20	1.80	2.60	4.60
	(b)	0.20 <sup>b</sup>	0.01	2.30	4.51

<sup>a</sup> Two different lots of casein were used to obtain this data. The data in the a and b experiments, indicative of the reproducibility of the results, were obtained with one lot of casein. The C-c, d results were obtained with a second lot of casein. Where — appears in the columns the particular solvent was not used.

<sup>b</sup> Used after petroleum ether.

in. The rest of the lipids (53-63%) are bound to the casein in such a manner that petroleum ether is unable to remove them. Acetone removes free lipids and some of the tighter bound lipids and CHCl<sub>3</sub>—CH<sub>3</sub>OH (2:1) can remove the rest (C-c,d). Thin-layer chromatography shows that petroleum ether extracts contain neutral fat (triglycerides, cholesterol, pigments, cholesterol esters, diglycerides, monoglycerides, and free fatty acids), but do not contain phospholipids in significant amounts. The CHCl<sub>3</sub>—CH<sub>3</sub>OH (2:1) extract (after petroleum ether extraction) (C-a,b) contains 45% petroleum ether-soluble lipids. This fraction consists of neutral fat (80%) and phospholipids (20%). The petroleum ether-insoluble portion consists of phospholipids and amino acids (after HCl hydrolysis). Acetone extract (after petroleum ether extraction) (C-c) contains phospholipids as a major component and neutral fat as a minor component. Acetone extraction removes neutral fat and phospholipids directly from freeze-dried casein (C-d).

Similar solvent effects are observed also with petroleum ether on freeze-dried whole milk. Petroleum ether extracts approximately 85% of neutral fats and no phospholipids or only traces. On the other hand acetone extracts from dried whole milk contain neutral fats and phospholipids. Presumably, the phospholipids and a part of neutral fats are bound to milk proteins in a manner that they are not readily extractable by petroleum ether but are extractable by acetone.

Thin-layer chromatography shows that acetone extracts and petroleum ether extracts from casein contain the same fat components as milk lipids obtained from whole milk but in different ratios. Carotenoid pigments, cholesterol esters, cholesterol, and phospholipid content of casein

lipids are relatively higher than in whole milk lipid.

The CHCl<sub>3</sub>—CH<sub>3</sub>OH (2:1) solvent alone removes practically all of the common lipids from dry casein. The CHCl<sub>3</sub>—CH<sub>3</sub>OH—HCl (200:100:1) solvent extracts 3.5 to 4.5% additional material from casein (on dry weight basis). The residue of the extract is a white solid containing 0.1-0.2% ether-soluble matter (neutral fats and phosphatides). The rest is soluble in water and 50% aqueous methanol and readily soluble in 0.1 N NaOH. The aqueous solutions are acidic. The solid contains 3.5% P and 13.5% N and shows positive reactions with ninhydrin and with fat reagents. The high N content suggests the presence of protein and the P content indicates the possibility of phosphatide-peptides (5,6,8). The amount of lipid obtained after saponification, presumably representative of covalently bound lipid, was relatively small, namely 0.3%, predominantly in the fatty acid fraction.

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