The Lipids of Collagen Preparations

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The purity of a soluble collagen preparation is usually controlled by determining the ratio of hydroxyproline nitrogen to total nitrogen. A ratio of 0.08, indicating the virtual absence of noncollagen protein, is usually attained with conventional purification procedures, which also remove most of the carbohydrates. Highly purified collagen preparations contain about 0.5 % hexose, which is an essential component of the collagen molecule.[1] It is a common experience that appreciable amounts of lipids are present in extracted rat skin collagens. The lipids interfere with the purification procedures and also with certain analytical work on collagen by giving rise to turbid extracts, which are difficult to clear by centrifugation. The purpose of the present work was to find out to what extent lipids are removed in the course of the conventional purification steps and how much and what kinds of lipids are present in the final collagen samples.

Experimental. The skins of 5-day-old and 2.5-year-old male rats were freed from hair and subcutaneous fat and homogenized separately for 15 sec with an Ultra-Turrax® homogenizer. Two soluble collagen fractions, NSC and ASC, were isolated by subsequent extraction with 0.45 M sodium chloride and 0.5 M acetic acid, respectively. These fractions were purified by repeated precipitation with sodium chloride and dialysis against alkaline buffers as described previously.[2] Tail tendons of the 2.5-year-old rats were extracted with 0.5 M acetic acid and the collagen was precipitated by dialysis against 0.01 M disodium phosphate and distilled water. Soluble collagens at different stages of purification and the insoluble collagen (ISC) were dialyzed against water, freeze-dried and weighed. The nitrogen[3] and hydroxyproline[4] contents of the raw extracts and final products were determined. The extracted lipids were purified[5] and analyzed for triglycerides,[6] cholesterol[7] and phosphorus.[8]

Considerable quantities of lipids are extracted together with soluble collagens from rat skin (Table 1). The dry non-dialyzable matter extracted by 0.45 M sodium chloride from the skins of both age groups contained approximately 13 % lipids. Still more (23 %) was present in the material subsequently extracted by 0.5 M acetic acid from the skins of the young rats, while appreciably less (1.0 %) was found in the corresponding fraction from the skins of the old animals. More lipid was left in the insoluble residue of the skins of the 5-day-old rats (19 %) than in that of the skins of the 2.5-year-old rats (4.3 %). These differences could be explained by the fact that the lipid content was higher in the skins of the younger animals (13 % of wet weight) than in the skins of the older animals (7 %). The material extracted by 0.5 M acetic acid from the tail tendons contained only 0.2 % lipids.

The conventional salt precipitation-dialysis techniques used to remove non-collagen proteins and carbohydrates fail to remove the lipids (Table 1). On the contrary, the percentage of lipids increases during these procedures, obviously as a result of their coprecipitation with collagen. The final NSC preparations from rat skins contained 31–32 % lipids, although they were pure as judged from the ratio of hydroxyproline nitrogen to total nitrogen.[9] The same applies to ASC isolated from the skins of the 5-day-old rats (36 % lipids), whereas the ASC from the skins of the 2.5-year-old rats was "purer" (1 % lipids).

The compositions of the lipids of the skin collagen samples resembled that of the lipids of whole skin: 93–94 % glycerides, 2–3 % cholesterol, and 3–5 % phospholipids. According to thin-layer chromatographic analyses, the glycerides were mainly triglycerides and the cholesterol was mostly unesterified; in addition, there were low proportions of free fatty acids. The increase in the lipid content during the purification procedure was mainly due to an increase in the triglycerides and to a lesser extent to an increase in cholesterol. The proportion of phospholipids remained constant or decreased slightly. The increase in the content of total lipids is obviously caused by removal of carbohydrates and non-collagen proteins; a part of the latter

Table 1. Lipid contents of rat skin collagen preparations at different stages of purification.

Preparation	5-day-old rats					2.5-year-old rats				
	Hypro N/ total N	Lipids, % of dry weight G [a]	Chol. [b]	P–L [c]	Total	Hypro N/ total N	Lipids, % of dry weight G [a]	Chol. [b]	P–L [c]	Total
Skin										
0.45 M NaCl-soluble:										
raw extract	0.02	11.0	0.84	1.72	13.5	0.03	10.9	0.82	0.94	12.6
after 1st precipitation with NaCl		15.3	1.20	1.17	17.6		21.2	1.66	0.85	23.7
after 2nd precipitation with NaCl		9.9	0.84	0.89	11.7		10.4	0.84	0.46	11.7
after 3rd precipitation with NaCl		18.5	1.05	1.10	20.6		12.2	1.04	0.42	13.7
after 4th precipitation with dialysis against alkaline buffers	0.07	28.0	1.72	2.11	31.8	0.08	28.8	1.70	0.69	30.6
0.5 M acetic acid-soluble:										
raw extract	0.02	19.0	1.33	2.73	23.1	0.07	0.57	0.03	0.01	1.0
after 1st precipitation with NaCl		14.5	1.27	2.01	17.8		0.13	0.19	0.02	0.3
after 2nd precipitation with NaCl		19.7	1.70	2.31	23.7		0.46	0.14	0.06	0.7
after 3rd precipitation with dialysis against alkaline buffers	0.09	31.5	1.87	2.77	36.1	0.08	1.28	0.07	0.02	1.4
Insoluble		13.7	1.52	3.38	18.6		3.86	0.25	0.19	4.3
Tail tendon										
0.5 M acetic acid extract							0.08	0.07	0.08	0.2
Precipitate							0.08	0.07	0.08	0.2

The lipids were extracted from freeze-dried samples with 100 volumes (v/w) of chloroform-methanol (1:1).
[a] glycerides, [b] cholesterol, [c] phospholipids

Table 2. The amounts of lipids extracted from freeze-dried NSC by different solvents.

Solvent	Extracted lipids, per cent of dry collagen		
	Glycerides	Cholesterol	Phospholipids
Hexane	2.6	0.13	<0.02
Benzene	2.9	0.18	<0.02
Diethyl ether	3.1	0.14	<0.02
Chloroform-methanol (1:1)	7.5	0.41	0.23
Methanol	7.0	0.38	0.31
Acetone	2.9	0.19	<0.02

Purified 0.45 M NaCl-soluble collagen from skins of 2.5-year-old rats was ground in a mortar and divided into six equal portions. Each portion was extracted with four 100-fold volumes (v/w) of solvent.

is probably lipoproteins which contain preferentially phospholipids and cholesterol.

The fact that polar solvents are required to remove lipids from the freeze-dried samples of soluble skin collagen (Table 2) indicates that the lipids are either strongly adsorbed to the protein or, more probably, occluded in a dense network of collagen fibres. Chloroform-methanol (1:1, v/v) and methanol were the most effective solvents in this respect. In order to determine the residual lipid content of the extracted collagen, a sample of purified NSC from the skin of a 2.5-year-old rat was extracted 3 times with 100 volumes (v/w) of chloroform-methanol (1:1) followed by a similar extraction with methanol, and transesterified with a 2 % solution of sulphuric acid in methanol at 70° for 8 h. The amount of fatty acid methyl esters extracted by hexane from the hydrolyzate was 1.8 μequiv./100 mg collagen, which corresponds to a residual lipid content of approximately 0.5 %.

Attempts were made to precipitate lipid-free collagen from its solutions with 5—10-fold volumes of chloroform-methanol or methanol. However, both solvents gave rise to scanty precipitates, which were difficult to collect by sedimentation or filtration. On the other hand, the addition of 5-fold volumes of either ethanol or ethanol-diethyl ether (3:1, v/v) to solutions in 0.5 M acetic acid of purified NSC from the skins of 2.5-year-old rats resulted in transparent and easily sedimentable precipitates, which dissolved rapidly in 0.5 M acetic acid. The recoveries of collagen were 90—97 % on the basis of hydroxyproline analyses. Most of the lipids (94 %) remained in the supernatant. The precipitates contained 0.4 % lipids (0.2 % glycerides, 0.1 % cholesterol and 0.02—0.07 % phospholipids) extractable with chloroform-methanol (1:1).

In conclusion it can be stated that different collagen preparations contain varying amounts of lipids which are not removed by conventional purification. procedures. When collagen is precipitated from its solution by a 5-fold volume of ethanol, most of the lipids remain in solution; the precipitated collagen dissolves readily in 0.5 M acetic acid. Whether lipids are structural components of collagen remains to be determined.

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