Lipids of mineralizing epiphyseal tissues in the bovine fetus

R. E. WUTHIER

Forsyth Dental Center, 140 The Fenway, Boston, Massachusetts 02115

ABSTRACT Because lipids had been consistently detected histologically at sites of new calcification, the lipids of epiphyseal cartilage and bone in various stages of mineralization were examined. Lipids were extracted before and after demineralization and analyzed. Lipid content increased during proliferation and calcification of epiphyseal cartilage. Much less was seen in the adjacent cancellous bone; this corroborates histochemical findings.

Similar phospholipid compositions were seen in the total lipids of cartilage and bone. Neutral (dipolar) phospholipids accounted for nearly 90% of the total lipid P and were almost completely extracted before demineralization. Serine- and inositol-containing phospholipids and two other, unidentified, acidic lipids could not be effectively extracted from calcifying tissues until after demineralization. Since the extraction of the acidic lipids was closely related to the degree of mineralization, it is possible that they form part of a lipoprotein–mineral complex in the calcifying matrix.

Lysophospholipids were detected in all extracts, but primarily in those made after decalcification. It is concluded that acidic lipids are mainly responsible for the sudanophilia detected histologically at sites of new calcification.

KEY WORDS lipids · epiphyseal cartilage · bone · phospholipids · lysophospholipids · acidic polar lipids · calcification · fetus

ALTHOUGH WELLS DISCUSSED the possible role of lipids in mineralization over 50 years ago (1), little attention has been directed toward the lipids of mineralizing tissues except in some studies of pathological calcification.

Recent interest in the lipids of calcified tissues has been aroused by the histological observations of Irving (2–7) and Irving and Wuthier (8) that sudanophilic (lipid) material was consistently found at sites of active calcification. In addition to being found in all normal calcifying tissues, this lipid material has been recently demonstrated at sites of ectopic calcification (Irving, 9). Enlow and Conklin (10) and Enlow, Conklin, and Bang (11) have also described the distribution of lipids in bone. These workers likewise suggested that there may be a relationship between the matrix lipid and the process of ossification.

The difficulties in analysis of small amounts of lipid have undoubtedly delayed any detailed investigation of the lipids present in calcifying tissues. Leach (12) reported briefly on the lipid content of untreated bovine compact bone. Zambotti, Cescon, Bonferroni, and Bolognani described the lipids of porcine epiphyseal cartilage (13). In their brief report the percentages of some lipid constituents and the fatty acid composition of the total lipid were described. More recently, Howell, Marquez, and Pita (14) described some phospholipids of normal and rachitic costal cartilate. This study dealt with phospholipids extracted without demineralization of the tissues. Similarly, Cruess and Clark (15) reported on some alterations in the lipids of bone caused by hypervitaminosis A and D and Dirksen (16) and Dirksen and Ikels (17) investigated the composition of the lipids in dentin. More recently Shapiro, Wuthier, and Irving (18) and Shapiro and Wuthier (19) reported on the phospholipids of developing fetal bovine dentin and dental pulp.

The present studies were undertaken to investigate the lipids present in epiphyseal cartilage, a primary goal being to identify the sudanophilic lipid at sites of the onset of new calcification. Because the Sudan black stains a narrow band in the extracellular matrix of hypertrophic cartilage, tissue from each stage of differentiation and mineralization of the epiphyseal plate was collected by careful dissection. Lipids were extracted with neutral lipid solvents before and after demineralization and then

Abbreviations: DEAE, diethylaminoethyl; TLC, thin-layer chromatography.

Solvent ratios are v/v.

finally with acidified lipid solvents, a procedure based in part on Irving's histochemical method.

METHODS

Dissection of Tissues

Joints from the leg bones of nearly full-term bovine fetuses, obtained immediately after slaughter, were chilled on ice and then frozen and stored at −70°C with solid CO_2. The bones were sawed into convenient pieces for dissection, thawed in 0.9% NaCl at 0–5°C, and dissected into six of the zones shown in Fig. 1: resting, proliferating, hypertrophic, and calcified cartilage, and cancellous and compact bone. The accuracy of separation of the individual zones was assessed by histological examination of both the dissected and the remaining undissected tissue at each stage of the dissection process.

The resting cartilage sample was typical embryonic hyaline cartilage and was essentially free from either perichondrial connective tissue, material from the secondary ossification center, or columnar cartilage. The proliferating cartilage sample consisted of differentiated columnar cartilage, contained no resting cartilage and a maximum of 10% hypertrophic cartilage. The samples of hypertrophic and calcified cartilage represented successive stages of mineralizing columnar cartilage for which no clear-cut line of demarcation could be made. The calcified cartilage sample was almost completely free of bone tissue and represented the completion of cartilagenous calcification.

Removal of Blood

Because resting (nonproliferating) cartilage and bone are tough and difficult to disintegrate, these tissues were refrozen in solid CO_2 and ground in a Wiley mill to pass a 10 mesh sieve (U.S. Standard Series). All tissues were then washed three times by gentle homogenization in a 0.9% NaCl solution (0–5°C) and three times with distilled water (0–5°C) to remove blood.

Lyophilization

After washing, tissues were freeze-dried in a Virtis lyophilizer operated at less than 5μ Hg pressure for 24 hr or longer to insure complete dehydration. The dried tissues were brought to atmospheric pressure under N_2 and weighed. The weights of the freeze-dried samples were: resting cartilage, 5.8–8.8 g; proliferating cartilage, 0.42–0.49 g; hypertrophic cartilage, 0.23–0.35 g; calcified cartilage, 1.13–1.57 g; and cancellous bone, 9.8–13.4 g. In order to obtain an accurate analysis of the minor lipid constituents, we dissected tissue from the various zones of the epiphyseal apparatus from several dozen fetal calf joints and pooled them. The weights of these samples were: resting cartilage, 36.3 g; proliferating cartilage, 4.82 g; hypertrophic cartilage, 4.73 g; calcified cartilage, 18.6 g; cancellous bone, 123.9 g; and compact bone, 89.5 g. In a separate experiment three sam-

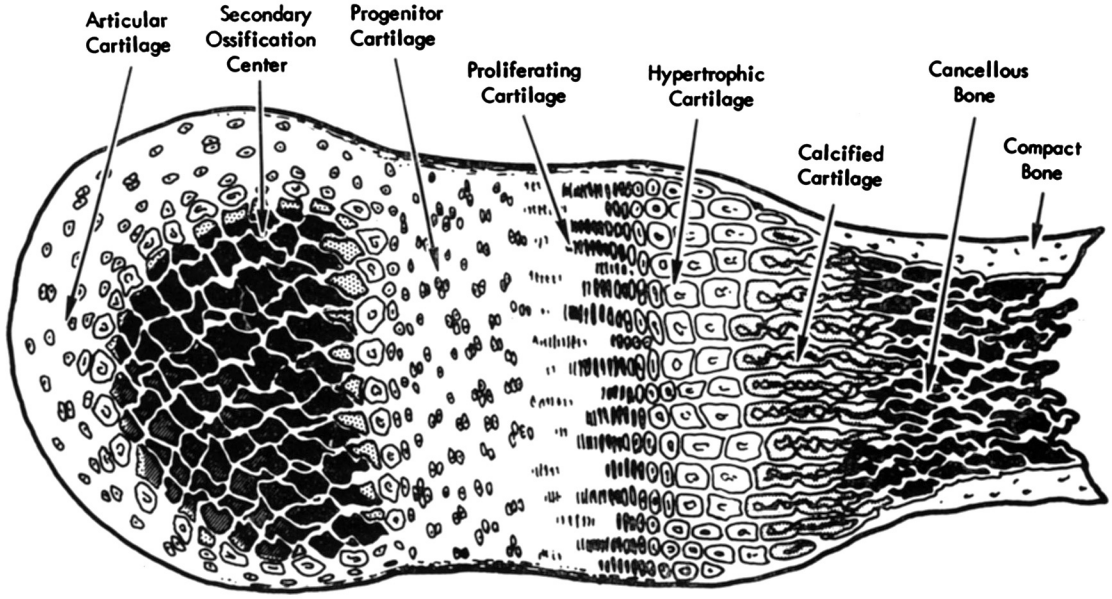


FIG. 1. Diagrammatic representation of the epiphyseal apparatus. Width of the epiphyseal plate and cell size are greatly exaggerated to permit identification of the various zones. Resting cartilage samples were obtained mainly from the articular cartilage area, rather than from the progenitor cartilage region. Great care was made to exclude from the resting cartilage samples any material from the secondary ossification center. Samples of the other cartilage zones were obtained by delicate scraping after fracturing the epiphysis between the hypertrophic and calcified cartilage zones. Cancellous bone and all other samples were carefully washed with 0.9% NaCl to remove blood and marrow.

ples (8.73–11.14 g, freeze-dried weight) of total epiphyseal cartilage consisting of proliferating (22%), hypertrophic (14%), and calcified cartilage (64%) were analyzed.

Extraction of Lipids (See Extraction Schemes 1 and 2)

Analytical grade solvents, redistilled in glass, were used. Dry tissues were homogenized in the solvents under an atmosphere of N_2 and allowed to stand for 24 hr at room temperature. For each subsequent extraction step, solvent and tissue remained in contact for 60 min. Chloroform–methanol 2:1 (20), 50–100 ml/g dry tissue, and chloroform–methanol–concentrated HCl 200:100:1 (21), 100 ml/g dry tissue, were used. Before being evaporated to dryness, acidic extracts were neutralized with dry $NaHCO_3$ and filtered. All drying was done under reduced pressure in an atmosphere of N_2.

Individual extracts were analyzed separately for lipid constituents, and the results were later combined to give the total.

Demineralization of Tissues (See Extraction Schemes 1 and 2)

Tissues were demineralized by treatment with at least a 5-fold excess (based on Ca content) of 0.5 M EDTA, pH 8.0 at 0–5°C. The dissected zones were demineralized for 48 hr and then dialyzed in cellophane tubing against cold (10–15°C) running tap water for 48 hr and distilled water (0–5°C) for 24 hr to remove the dissolved mineral and decalcifying agent. The nondialyzable material was collected, lyophilized, weighed, and further extracted (Scheme 1). Because the results from the studies of tissue zones indicated that lipid was being hydrolyzed and lost during the demineralization and dialysis steps, the total epiphyseal cartilage samples were demineralized for only 16 hr. The tissue was then centrifuged at 0–5°C for 30 min at 10,000 *g* to sediment EDTA-insoluble materials. The supernatant fraction and precipitate were collected separately, dialyzed against running tap water (5–10°C) for 16 hr, lyophilized, weighed, and further extracted for lipids (Scheme 2). By using shorter exposure to aqueous solutions and by examining both the EDTA-soluble and -insoluble material, we hoped that some evaluation of the extent of degradation and loss of lipids during the demineralization procedure would be possible.

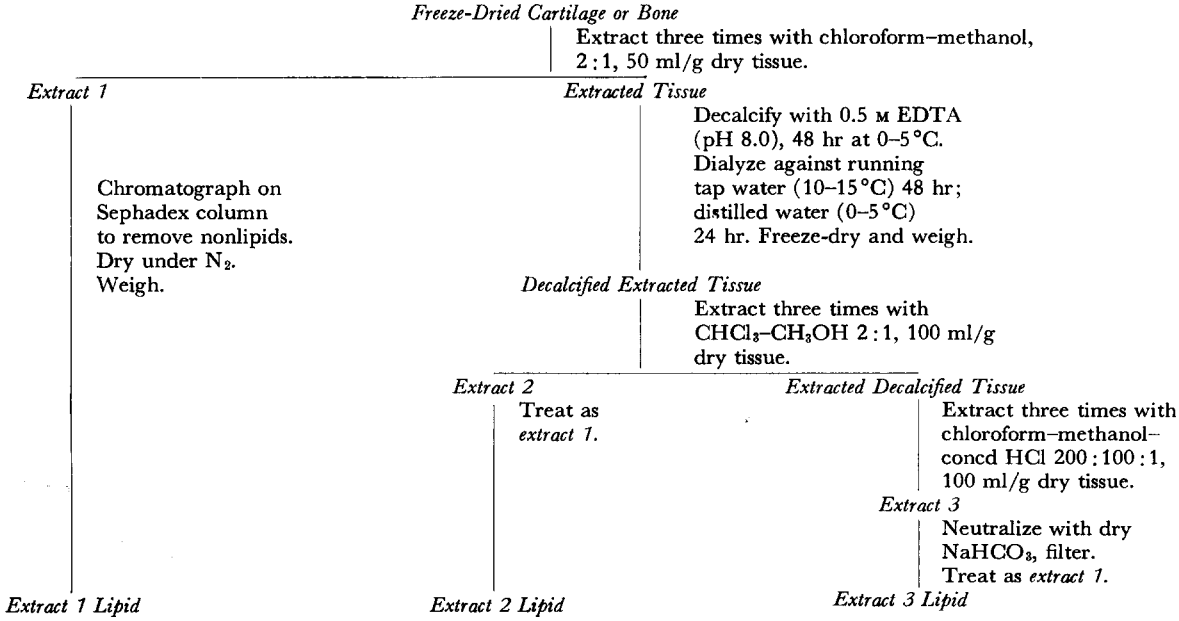
Purification of Lipid Extracts

Water-soluble nonlipid contaminants were removed by Sephadex column chromatography as previously reported (22). The eluate containing the purified lipid was taken to dryness under reduced pressure in an atmosphere of nitrogen, transferred with chloroform to preweighed vials, dried under N_2 under high vacuum (<5μ Hg) over paraffin shavings for 24 hr, and weighed.

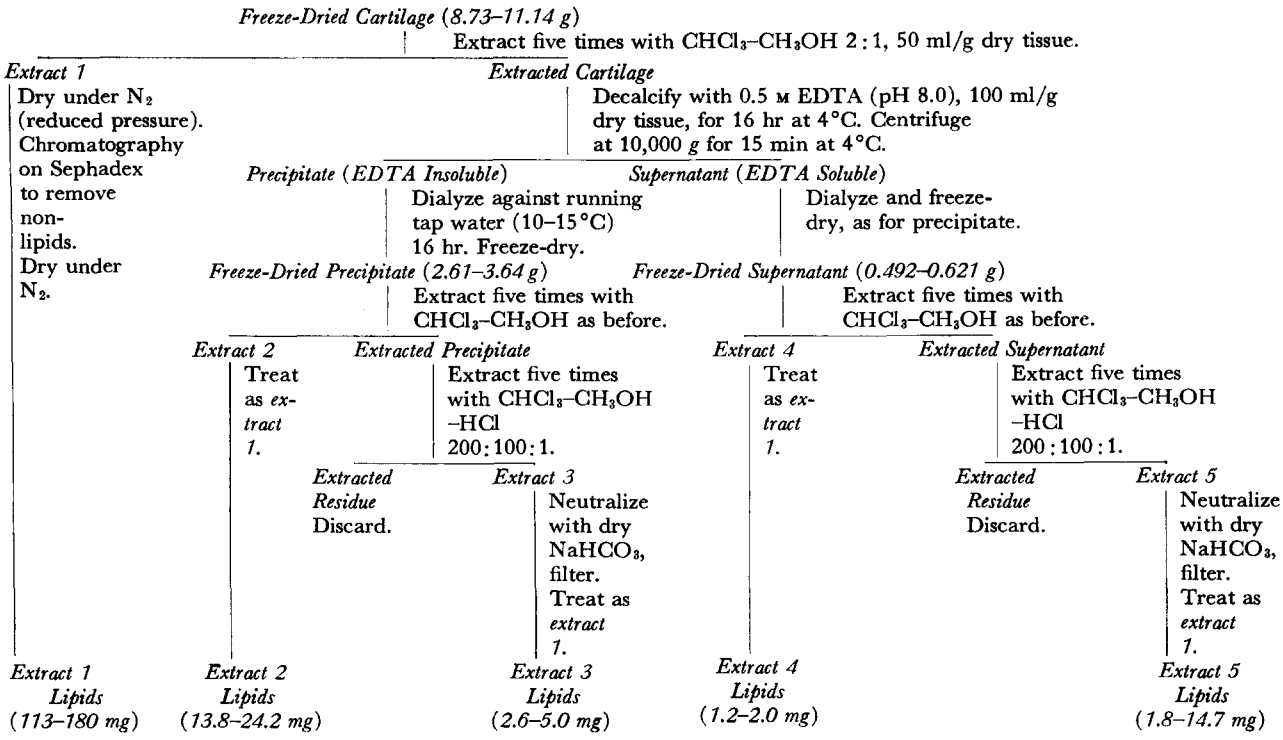
Separation of Polar and Nonpolar Lipid Fractions

Polar and nonpolar lipids were separated by applying a chloroform solution of the purified lipids to columns (1 cm I.D. × 10 cm) of silicic acid (Mallinckrodt, 100 mesh, A.R.), previously washed with chloroform–methanol 2:1 followed by pure chloroform. Nonpolar lipids were eluted with 25 ml of chloroform, and polar lipids with 50 ml of methanol.

SCHEME 1 TISSUE EXTRACTION OF EPIPHYSEAL CARTILAGE ZONES



SCHEME 2 TISSUE EXTRACTION OF TOTAL EPIPHYSEAL CARTILAGE



Identification of Individual Phospholipids

The identity of phospholipids separated by two-dimensional paper chromatography (23) was verified as follows. The polar lipid fractions were subjected to DEAE-cellulose column chromatography (24), which gave choline-, ethanolamine-, and serine-containing phospholipids, phosphatidic acid-like phospholipids, and the remaining asidic phospholipids. The DEAE column fractions were monitored by two-dimensional paper chromatography and then further fractionated on silicic acid columns (25) or by preparative TLC on silica gel in the solvent systems of Müldner, Wherrett, and Cumings (26) and Skipsi, Peterson, and Barclay (27) to separate the individual types of choline-, ethanolamine-, serine-, and inositol-containing phospholipids. For diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidic acid, chloroform–methanol–1 N NH_4OH 80:20:2 was used as the solvent for TLC. The isolated fractions were chromatographed on Sephadex G-25 columns to remove non-lipid materials (22), examined for purity by paper chromatography, and then examined as thin films by IR spectrophotometry using a Perkin-Elmer, model 137B spectrophotometer equipped with a beam condenser. The individual lipid fractions (and phospholipid standards) were further subjected to mild alkaline hydrolysis (28) and the deacylated products were chromatographed in the solvent systems of Dawson (29, 30).

Quantification of Individual Lipids

Polar lipids were separated by two-dimensional chromatography on silica gel-loaded paper and the phospholipids quantified by measuring phosphorus content of the individual chromatographic components (23).

Determination of Moisture and Ash

For moisture determination, samples of freshly dissected tissue were immediately weighed in tared polyethylene containers. The containers were unsealed and the contents were frozen in situ over solid CO_2 and freeze-dried overnight. Dried samples were transferred to a desiccator containing anhydrous silica gel. The desiccators were evacuated to a pressure of less than 5μ Hg for 2 hr and refilled with dry N_2 at atmospheric pressure; the polyethylene containers were resealed and weighed. For ash analysis, samples of the dried tissue were slowly heated in platinum crucibles to 600°C, ashed overnight, cooled to room temperature in a desiccator containing anhydrous silica gel, and weighed.

RESULTS

Moisture and Ash

Percentages of moisture, mineral, and organic matter in the different calcifying zones are shown in Table 1.

Moisture content was greatest in proliferating cartilage and decreased as mineralization progressed. Nondialyzable organic matter represented the bulk of the original dry weight of resting cartilage, but only about one-fifth that of calcified cartilage.

Total Lipids

Table 2 presents the total lipid, polar and nonpolar lipid, and total lipid phosphorus extracted from the various epiphyseal zones. Because of the widely different moisture, mineral, and organic contents of the calcifying tissues, total lipids are presented as wet, dry, and demineralized dry weight. As shown in Table 2, the lipid content of cartilage steadily increased as mineralization proceeded, if it is expressed as either wet or demineralized dry weight. Bone had values similar to those of resting cartilage. When expressed as a percentage of the original dry weight, lipid content was highest in proliferating cartilage. This undoubtedly reflected the higher moisture content of this tissue zone. The content of nonpolar lipids was highest in hypertrophic cartilage; the content of polar lipids increased progressively with mineralization of the cartilage. Cancellous bone had a polar lipid content only one-twentieth of that in the adjacent calcified cartilage zone. Highest values for total lipid P were found in proliferating and calcified cartilage. Values for lipid P of bone were much lower, similar to those in resting cartilage.

Yield of Lipids from Sequential Extractions of Tissues

The yields of total lipid and lipid P from the sequential extractions of the various zones and of the total epiphyseal cartilage are presented in Tables 3 and 4. In the total epiphyseal cartilage (Table 3) only 80.6% of the total lipid and 87.3% of the total lipid P could be extracted before demineralization (*extract 1*, Scheme 2). Extraction with neutral chloroform–methanol after demineralization (*extracts 2* and *4*, Scheme 2) removed most of the remaining lipid; however, over 7% of the total lipid and nearly 2% of the lipid P were not removed until the tissues were extracted with acidified solvent (*extracts 3* and *5*, Scheme 2). Most of the lipid and nearly all of the lipid P not extracted before demineralization were found in the EDTA-insoluble residue (Table 3).

TABLE 1 COMPOSITION OF EPIPHYSEAL TISSUES

Zone	Moisture	Ash: % of Wet Weight	Ash: % of Dry Weight	Nondialyzable Organic Matter*: % of Wet Weight	Nondialyzable Organic Matter*: % of Dry Weight
	% wet wt				
Resting cartilage	84.5 ± 0.4 (4)	0.79	5.1 ± 0.3 (16)	14.5	93.9 ± 5.8 (3)
Proliferating cartilage	88.0 ± 0.4 (4)	1.66	13.2 ± 0.8 (7)	7.65	62.8 ± 2.0 (3)
Hypertrophic cartilage	82.5 ± 0.2 (4)	9.39	56.0 ± 1.5 (11)	5.88	33.6 ± 3.4 (5)
Calcified cartilage	55.8 ± 0.9 (4)	30.6	69.3 ± 0.6 (12)	8.22	18.6 ± 1.3 (5)
Cancellous bone	32.9 ± 0.3 (4)	42.2	64.6 ± 0.6 (11)	16.5	24.6 ± 1.2 (2)
Compact bone	30.3 ± 1.2 (5)	42.1	60.4 ± 1.0 (8)	18.7	26.8 (1)

Values are expressed as means ± SEM; the figures in parentheses are the number of samples.
* Nondialyzable organic matter is the residue after dialysis of the demineralized tissue.

TABLE 2 TOTAL EXTRACTABLE LIPID FROM EPIPHYSEAL TISSUES

Calcification Zone	Number of Samples	Total Lipid as % of Tissue Weight: Wet	Total Lipid as % of Tissue Weight: Dry	Per Demineralized Dry Weight: Total Lipid	Nonpolar Lipid	Polar Lipid	Total Lipid P
				% by weight			*mg/100 g*
Resting cartilage*	3	0.15	0.95 ± 0.16	1.00 ± 0.12	0.47 ± 0.06	0.53 ± 0.11	14.3 ± 1.7
Proliferating cartilage*	3	0.36	2.98 ± 0.46	4.86 ± 0.90	2.07 ± 0.40	2.79 ± 0.51	66.2 ± 5.4
Hypertrophic cartilage*	5	0.43	2.44 ± 0.34	7.70 ± 1.49	4.33 ± 0.91	3.37 ± 0.71	60.9 ± 3.7
Calcified cartilage*	5	0.68	1.57 ± 0.14	8.50 ± 0.81	2.98 ± 0.28	5.52 ± 0.69	111.9 ± 5.0
Cancellous bone*	4	0.12	0.21 ± 0.02	0.61 ± 0.06	0.33 ± 0.14	0.28 ± 0.04	6.1 ± 1.6
Compact bone*	1	0.16	0.23	0.98	—	—	13.2
Total epiphyseal cartilage†	3	—	1.72 ± 0.17	4.65 ± 0.59	—	—	76.1 ± 8.2

The values for total extractable lipids, nonpolar lipid, and lipid P were determined from separate analyses of *extracts 1*, *2*, and *3* by summation. Nonpolar lipids are those eluted from silicic acid columns with chloroform. Polar lipids are those retained during chloroform elution, but eluted with methanol. Values are means ± SEM. No estimate of variability is given for values expressed on wet weight because they were derived.
* Tissues extracted according to Scheme 1.
† Tissues extracted according to Scheme 2.

TABLE 3 YIELD OF TOTAL LIPID AND LIPID P IN SEQUENTIAL EXTRACTIONS OF TOTAL EPIPHYSEAL CARTILAGE

Fraction*	Lyophilized Weight†	Extract*	Lipid		Lipid P		
			Wt†	% of Total‡	Wt†	Fraction of lipid‡	% of Total‡
	g		*mg*		*μg*	*μg/mg*	
Before demineralization	10.24 (8.73–11.14)	1	142 (113–180)	80.6 ±2.7	2600 (1570–3185)	18.2 ±2.9	87.3 ±3.9
After demineralization							
EDTA-insoluble	3.25 (2.61–3.64)	2	19.3 (13.8–24.2)	11.0 ±1.3	281 (149–366)	14.2 ±1.7	10.6 ±3.6
		3	4.1 (2.6–5.0)	2.35 ±0.39	40 (33–53)	10.0 ±1.7	1.39 ±0.22
EDTA-soluble	0.544 (0.492–0.621)	4	1.7 (1.2–2.0)	0.95 ±0.10	12 (6–18)	7.1 ±1.2	0.41 ±0.07
		5	9.2 (1.8–14.7)	5.04 ±1.98	8 (6–12)	1.9 ±1.1	0.33 ±0.14
Total			168 (147–226)	100.00	2941 (1948–3488)	16.0 ±3.2	100.00

* See Scheme 2 for tissue extraction.
† Mean of three samples (range of values).
‡ Mean of three samples ± SEM.

TABLE 4 YIELD OF LIPID PHOSPHORUS IN SEQUENTIAL EXTRACTION OF CALCIFIED TISSUES

Calcification Zone	Number of Samples	Lipid Phosphorus			
		Extract 1	*Extract* 2	*Extract* 3	Total
		mg/100 g demineralized tissue			
Resting cartilage	3	7.2 ± 0.7	6.7 ± 1.0	0.5 ± 0.1	14.3 ± 1.7
Proliferating cartilage	3	61.5 ± 2.2	3.9 ± 2.3	0.8 ± 0.1	66.2 ± 5.4
Hypertrophic cartilage	5	52.6 ± 4.1	6.6 ± 1.2	1.7 ± 0.6	60.9 ± 3.7
Calcified cartilage	5	100.0 ± 3.5	9.2 ± 1.2	2.6 ± 0.9	111.9 ± 5.0
Cancellous bone	4	5.2 ± 1.2	0.4 ± 0.2	0.5 ± 0.2	6.1 ± 1.6
Compact bone	1	9.4	2.4	1.3	13.2

See Scheme 1 for tissue extraction. *Extract 1* was made before demineralization; *extracts 2* and *3* after. Values are means ± SEM.

The yields of lipid P from the sequential extractions of the various zones of calcification are presented in Table 4. With the exception of resting cartilage, 86–93% of the total lipid P of cartilage and 71–85% of the total lipid P of bone were extracted before demineralization (*extract 1*, Scheme 1); 5.9–10.9% of the total lipid P of cartilage and 7.2–18.5% of the total lipid P of bone were removed with neutral chloroform–methanol after demineralization (*extract 2*, Scheme 1); and 1.2–3.5% of the total lipid P of cartilage and 7.5–10.1% of the lipid P of bone were released with acidified chloroform–methanol (*extract 3*, Scheme 1). In resting cartilage only half of the lipid P was extracted before treatment with EDTA. This appeared to be the result of impaired diffusion of the lipid solvent into this tissue (see Discussion).

Phospholipid Composition of the Total Extractable Lipid

Similar phospholipid compositions were seen in the total extractable lipid P from cartilage and bone in various stages of calcification (Tables 5 and 6). Neutral (dipolar) phospholipids represented 85–90% of the total lipid P, the acidic phospholipids representing the remainder. In proliferating cartilage, ethanolamine phospholipids were present in significantly higher proportions than in resting cartilage, apparently at the expense of phosphatidyl choline and sphingomyelin. Bone phospholipids were similar to those of cartilage except that the proportions of sphingomyelin and serine phospholipids were significantly higher. Small amounts of unidentified acidic and neutral phospholipids were found in both cartilage and bone.

Significant amounts of lysophospholipids were found in all zones (Table 5), but especially in the samples of total epiphyseal cartilage (Table 6). The proportion of lysophosphatidyl ethanolamine was highest in resting cartilage and decreased progressively with increasing mineralization. Lysophosphatidyl serine was found only in calcifying tissues.

Phospholipid Composition of the Sequential Extracts

In extracts made before demineralization (*extract 1*), neutral phospholipids represented the greatest bulk of the lipid P, especially in the calcifying tissues where in compact bone, for example, they accounted for over 98% of

TABLE 5 DISTRIBUTION OF LIPID PHOSPHORUS AMONG TOTAL PHOSPHOLIPIDS OF CALCIFYING TISSUES

Phospholipid	Cartilage				Bone	
	Resting	Proliferating	Hypertrophic	Calcified	Cancellous	Compact
	*% of total lipid P**					
A. Neutral						
Sphingomyelin	6.3 ± 0.4	5.0 ± 0.6	5.9 ± 0.7	6.0 ± 1.2	10.8 ± 0.8	9.1
Phosphatidyl choline	61.3 ± 1.3	55.2 ± 3.5	61.3 ± 1.5	55.6 ± 0.9	52.2 ± 1.8	58.0
Lysophosphatidyl choline	2.5 ± 1.1	1.3 ± 1.0	0.8 ± 0.5	2.0 ± 0.3	3.4 ± 0.4	1.3
Phosphatidyl ethanolamine	15.3 ± 1.1	26.0 ± 1.3	19.3 ± 0.9	23.9 ± 1.0	17.5 ± 1.6	18.6
Lysophosphatidyl ethanolamine	4.0 ± 1.0	2.6 ± 1.8	1.5 ± 0.8	0.6 ± 0.3	1.1 ± 0.6	1.3
Unidentified neutral phospholipids	0.0	0.0	0.0	0.0	0.2 ± 0.0	0.0
Total neutral phospholipids	89.2 ± 0.5	88.9 ± 2.7	88.6 ± 1.8	87.1 ± 1.3	85.2 ± 1.1	88.3
B. Acidic						
Phosphatidyl serine	3.7 ± 0.5	3.2 ± 0.1	2.6 ± 0.3	4.2 ± 0.7	5.5 ± 0.5	6.5
Lysophosphatidyl serine	0.0	0.0	0.4 ± 0.3	0.3 ± 0.1	1.0 ± 0.2	0.3
Phosphatidyl inositol	4.1 ± 0.4	4.0 ± 0.5	2.6 ± 0.4	3.2 ± 1.0	3.0 ± 0.8	1.1
Diphosphoinositide (tent.)	0.2 ± 0.1	0.3 ± 0.5	0.7 ± 0.4	0.4 ± 0.1	0.0	0.0
Phosphatidic acid	0.5 ± 0.0	1.3 ± 0.4	1.6 ± 0.6	0.5 ± 0.1	1.4 ± 0.4	0.8
Diphosphatidyl glycerol	0.8 ± 0.1	1.2 ± 0.3	1.4 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	1.5
Phosphatidyl glycerol	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.4
"Chondrolipin"†	0.1 ± 0.0	0.4 ± 0.2	1.2 ± 0.3	1.0 ± 0.1	0.5 ± 0.2	0.0
Unidentified acidic phospholipids	0.9 ± 0.3	0.8 ± 0.5	0.6 ± 0.2	1.0 ± 0.7	1.6 ± 0.6	1.2
Total acidic phospholipids	10.8 ± 0.2	11.1 ± 1.1	11.4 ± 0.8	12.9 ± 1.2	14.8 ± 1.2	11.7
Number of samples	3	3	5	5	4	1

Each phospholipid includes both the diacyl and alkenyl-acyl forms. Percentage of the total lipid P was obtained by summation of P from each component on silica gel-loaded paper chromatograms, the recovery of total lipid P applied to chromatograms being 93.5–99.5%. Values are means ± SEM.

* Total lipid P is the summation of amounts from *extracts 1*, *2*, and *3*.

† This lipid may be a degraded form of phosphatidyl inositol (see Discussion).

TABLE 6 PHOSPHOLIPID OF TOTAL EPIPHYSEAL CARTILAGE LIPID FRACTIONS

Phospholipid	Before Demineralization	After Demineralization				
		EDTA-Insoluble		EDTA-Soluble		
	*Extract 1**	*Extract 2*	*Extract 3*	*Extract 4*	*Extract 5*	Total Lipid P†
A. Neutral						
Sphingomyelin	3.4	5.1	6.7	5.8	8.4	3.7 ± 0.6
Phosphatidyl choline	46.5	31.3	25.7	25.0	23.9	44.9 ± 3.7
Lysophosphatidyl choline	6.0	3.0	5.6	7.1	4.6	5.5 ± 2.3
Phosphatidyl ethanolamine	27.7	14.6	7.9	7.7	5.6	26.0 ± 3.0
Lysophosphatidyl ethanolamine	3.3	8.6	8.4	7.3	6.8	4.1 ± 1.3
Unidentified neutral phospholipids	0.5	1.6	1.6	2.0	0.0	0.6 ± 0.3
Total neutral phospholipids	87.5	64.2	55.9	54.9	49.3	84.7 ± 1.0
B. Acidic						
Phosphatidyl serine	3.4	15.8	11.9	9.3	2.5	4.6 ± 0.2
Lysophosphatidyl serine	0.4	8.4	4.3	15.6	0.0	1.6 ± 0.8
Phosphatidyl inositol	1.3	3.4	1.6	5.4	1.1	1.4 ± 0.6
Diphosphoinositide (tent.)	0.0	0.4	0.9	1.0	0.0	0.1 ± 0.0
Phosphatidic Acid	0.7	2.2	1.6	3.5	0.5	0.8 ± 0.1
Diphosphatidyl glycerol	2.4	1.6	3.4	3.6	5.5	2.3 ± 0.2
Phosphatidyl glycerol	0.7	0.8	1.4	1.6	0.0	0.7 ± 0.1
"Chondrolipin"‡	0.6	0.0	9.9	0.0	23.2	0.8 ± 0.6
Unidentified acidic phospholipids	3.0	2.8	8.6	4.0	17.7	3.2 ± 0.7
Total acidic phospholipids	11.8	35.5	43.8	44.0	50.5	15.3 ± 1.0

* See Scheme 2 for tissue extraction.

† Mean of three samples ± SEM.

‡ May be a degraded form of phosphatidyl inositol (see Discussion).

the lipid P. The percentage of acidic phospholipids in this extract was inversely related to the degree of mineralization of the tissue (Fig. 2). Serine and inositol phospholipids in particular represented a progressively smaller proportion of the lipid P extracted without demineralization (Fig. 3, columns C and D). However, diphosphatidyl glycerol and phosphatidyl glycerol were almost completely extracted before demineralization.

In extracts made with neutral chloroform–methanol after demineralization (*extract 2*, Scheme 1), the percentage of acidic phospholipids increased progressively in proportion to the degree of prior mineralization of the carti-

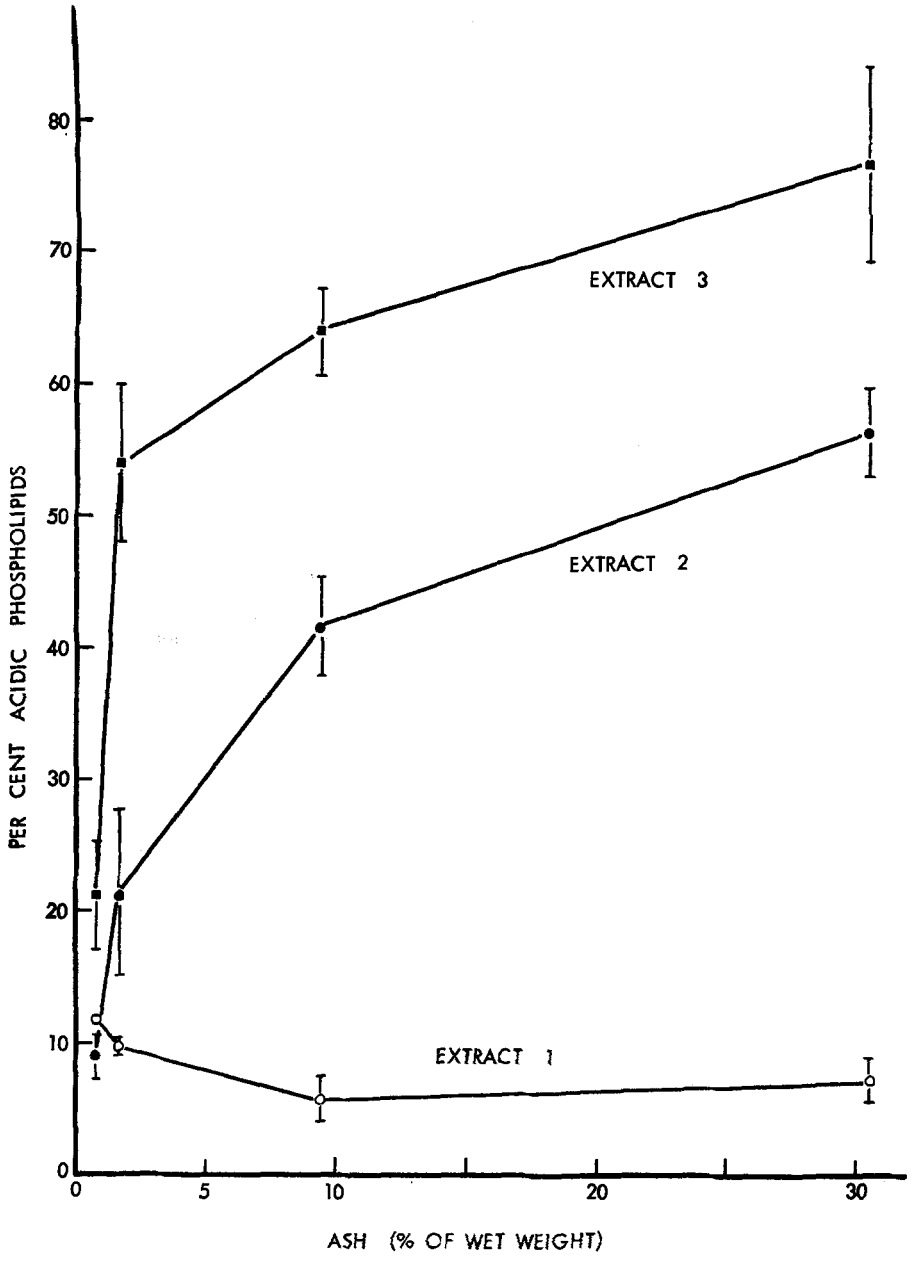


FIG. 2. Changes in acidic phospholipid content of sequential extracts of mineralizing cartilage. *Extract 1* was made with chloroform–methanol 2:1 before demineralization; *extract 2* with the same solvent after demineralization; and *extract 3* with chloroform–methanol–concentrated HCl 200:100:1 after that (see Scheme 1).

Note the direct relationship between the ash content of the tissues and the proportion of acidic phospholipids in extracts made after demineralization. Note also the inverse relationship between the ash content and the proportion of acidic phospholipids in extracts made before demineralization. Acidic phospholipid is the sum of the individual acidic phospholipids present in each extract. Experimental points show the mean and SEM of three to five samples.

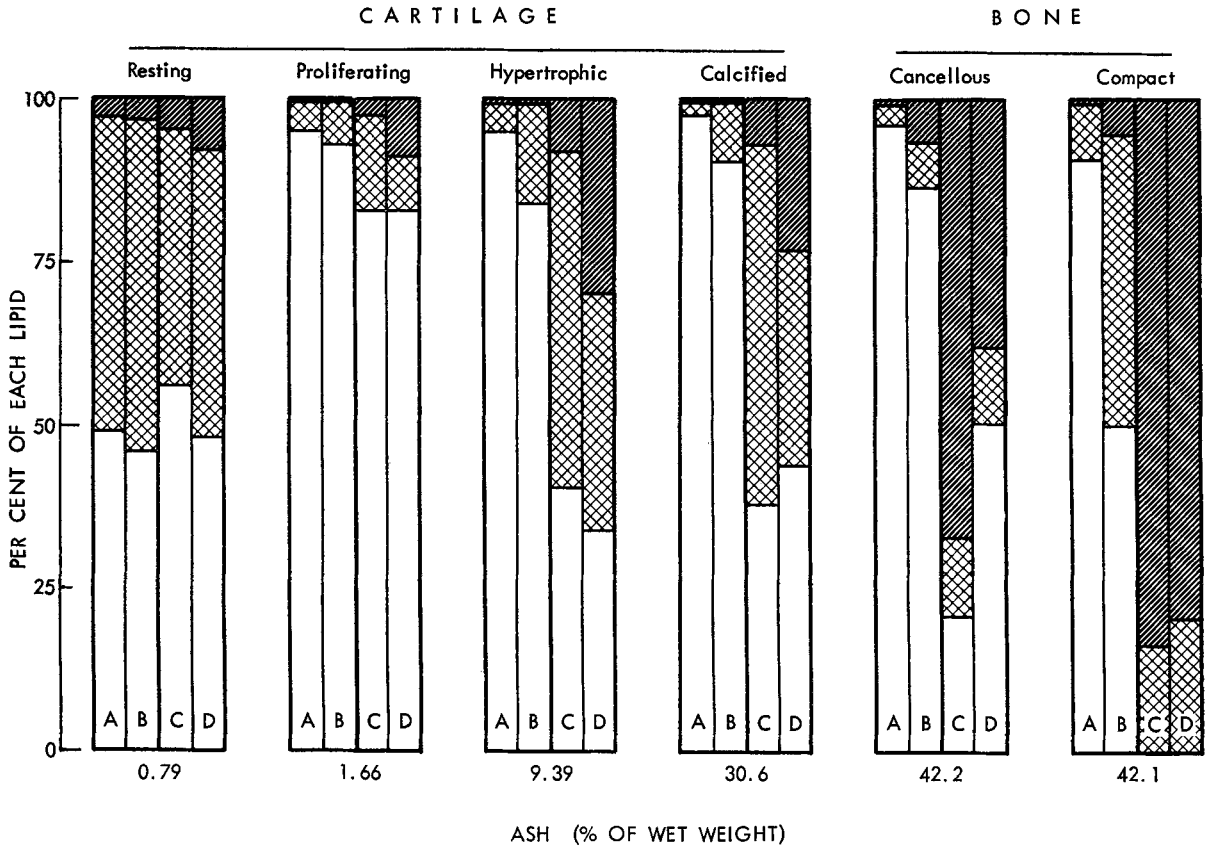


FIG. 3. Relationship between extractability of neutral and acidic phospholipids and degree of calcification. A, choline phosphoglycerides; B, ethanolamine phospholipids; C, serine phospholipids; D, inositol phospholipids (including "chondrolipin"). Within each column, the clear area represents lipid removed before demineralization (*extract 1*); the cross-hatched area, lipid removed after demineralization (*extract 2*); and the diagonal-hatched area, lipid removed with acidified chloroform–methanol (*extract 3*, Scheme 1).

Note the marked decrease in extractability of acidic phospholipids (columns C, D) in zones with appreciable calcium content. In these zones, from 50 to 100% of the serine- and inositol-containing phospholipids could be extracted only after demineralization. In bone, little of either of these lipids was extracted until acidified solvent was employed.

lage (Figs. 2 and 3). Serine and inositol phospholipids were the major acidic phospholipids, representing 29.8 and 18.5% of the lipid P from calcified cartilage. The proportion of lecithin was inversely related to the degree of prior calcification of cartilage. The proportion of sphingomyelin in bone was increased in this extract. Ethanolamine phospholipids represented 20–30% of the lipid P extracted from all tissues by this treatment except for compact bone where they represented nearly half of the lipid P.

Chromatograms of the final acidified solvent extract (*extract 3*, Scheme 1) revealed many lipid components, a considerable number of which have not been identified. The most characteristic feature of these extracts (Fig. 3) was the high proportion of acidic lipids. This was directly related to the extent of prior mineralization, i.e., resting cartilage had the smallest proportion of acidic lipids; compact bone the largest. Two acidic lipids contributed the major portion of the lipid P in these extracts: in cartilage "chondrolipin" accounted for nearly half of the lipid P; in bone, serine phospholipids accounted for over half of the lipid P.

Similar results were seen in the sequential extracts of total epiphyseal cartilage (Table 6). However, significantly higher amounts of lysophospholipids, especially lysophosphatidyl serine, were seen in extracts made after decalcification.

An unidentified acidic lipid component which did not contain phosphorus was consistently found in extracts made after demineralization. Small amounts of this lipid have been isolated in chromatographically pure form. From a variety of chemical, chromatographic, and spectrophotometric analyses, we concluded that it was not one of the common lipids (i.e. glycerides, sterols, or sphingolipids). It is possible that it may be related to one of the prostaglandins.

DISCUSSION

The analysis of lipids in calcified tissues presented several

problems not encountered in soft tissues. These tissues were difficult to thoroughly disintegrate and required freezing and grinding in a mill in order to obtain adequate reduction. In addition, since significant amounts of lipid were not extracted unless the tissue was freed of its calcium deposits, considerable exposure of the tissue to aqueous media was required. Although EDTA was selected as the decalcifying agent least liable to produce lipid degradation, some hydrolysis of lipid probably occurred during the decalcification procedure. Although it is likely, therefore, that the results reported for some lipids may be somewhat lower than the true tissue content, they do nevertheless offer a reasonable estimate of the lipids present in these tissues.

Significant amounts of lysophospholipids were found in nearly all of the extracts from the tissue zones. When attempts were made to assess the formation and loss of these lipids by using the total epiphyseal cartilage (Scheme 2) and by reducing the exposure to aqueous solutions, significantly greater amounts of acidic lipids (mainly phosphatidyl serine) and lysophospholipids were found. Since some lysophospholipid was found in the EDTA supernatant solution (Table 6, *extracts 4* and *5*), some loss (particularly of the acidic lipids) probably occurred during the demineralization and dialysis of the samples from the various zones of calcification. Some workers consider that lysophospholipids are artifacts and that those found in tissues are due to autolysis after death. Accordingly, it seemed possible that these lipids were formed during the demineralization and dialysis of the tissue, despite the fact that temperatures were maintained as low as possible. However, recent studies on epiphyseal cartilage of fowl using ^{32}P (Eisenberg, Wuthier, and Frank, unpublished) have shown that the specific activities of some lysophospholipids were significantly higher than those of the "parent" phospholipids. This suggests that the lysophospholipids probably were not artifacts of preparation. It is of interest here that Burstone (31) demonstrated histochemically that a high level of esterase activity exists in epiphyseal cartilage undergoing calcification. Nevertheless, since the labeling of the more stable ether forms of phospholipid may be slower than that of the more labile diester form, this may account for the discrepancy in the ^{32}P labeling.

Freeze-drying of the tissues, which was done to obtain accurate initial dry weights, caused only minor alteration in lipid extraction. Recent unpublished studies using fresh chicken and calf cartilage revealed that the basic pattern of extraction was very similar to that of the freeze-dried tissues—that is, neutral phospholipids were almost completely extracted before demineralization, whereas acidic lipids required decalcification of the tissue for complete extraction. However, very little "chondrolipin" (23) was found in extracts of fresh tissue. Since "chondrolipin" was extracted from freeze-dried calcifying cartilage only in the final stages when acidified solvents were employed, it could have been an artifact; on the other hand, freeze-drying of the tissue may actually be required for liberation of this lipid. Because of the limited amounts of material available, this lipid has not been completely characterized. Microbiological assays on acid hydrolysates of "chondrolipin" indicated that they did not contain myoinositol; however, chemical tests showed that they did have a large periodate consumption. Upon mild alkaline hydrolysis the deacylation product (30) migrated differently on chromatography and electrophoresis from the products of known phospholipids. Similarly, its IR spectrum differed from that of known phospholipids (24). The P content of the lipid was slightly less than 4%.

The lipid contents of the calcifying tissues were quite low when compared to those of nervous tissue, viscera, or muscle. This difference was undoubtedly due to the large amount of extracellular material present in the calcifying tissues. Nevertheless, a 4- to 6-fold increase in lipid content occurred during the transformation from resting to calcified cartilage. Since the composition of the cartilage was distinctly different from serum lipids, the observed increase in lipid content was probably due to a net synthesis of lipid, rather than an accumulation from circulating lipid.

Despite the general similarity in the proportions of the total acidic and neutral phospholipids, certain significant differences in the proportions of individual lipids did appear between cartilage and bone. For example, bone had a higher proportion of sphingomyelin and serine phospholipids, and a decreased proportion of inositol and ethanolamine phospholipids. There was a marked increase in ethanolamine phospholipids in proliferating cartilage compared with resting cartilage. These findings, taken with those of Howell et al. (14) which revealed a decreased proportion of ethanolamine phospholipids in rachitic cartilage, suggest that the synthesis of these lipids may be necessary for normal mineralization of cartilage.

The analyses of extracts made before and after demineralization of the calcifying tissues were of special interest. Most lipids of mammalian tissues are readily extracted with chloroform–methanol (32) if adequate contact with the solvent is provided by thorough disintegration of the tissue and repeated extraction, the exceptions being that polyphosphoinositides of brain (33) and phosphatidopeptides (34) require acidified solvents for effective extraction. The failure, therefore, to extract all of the lipids from *resting* cartilage before treatment with EDTA would appear to be due to the imperviousness of the cartilage. This was substantiated

by the finding (Fig. 3) that there was relatively little difference between the lipid compositions of the three sequential extracts from this tissue. In contrast, the failure to remove all of the lipids from *calcifying* tissues before demineralization appeared to be due to an interaction between certain of the acidic lipids, the newly forming mineral, and the protein–polysaccharides of the matrix (35). This was suggested by the distinct changes in this case in the proportions of the acidic and neutral phospholipids from the three sequential extracts (Fig. 3), which was directly related to the ash content of the tissue. It was further supported by the close relationship between the extractability of acidic lipids (especially serine and inositol phospholipids) and the extent of mineralization (Fig. 2). It is also consistent with the findings of Abramson and coworkers (36, 37), Tobias and coworkers (38, 39), Hendrickson and Fullington (40), and others that certain acidic phospholipids possess strong affinity for calcium.

The finding that significant amounts of lipid were refractory to extraction until the tissues were decalcified (Table 3) suggests that a stable complex, probably of protein–polysaccharide, lipid, and amorphous calcium phosphate (41), must have occurred in the matrix of tissues undergoing calcification. The additional release of lipid upon extraction with acidified lipid solvents suggests that direct salt-like linkage probably occurred between the matrix protein and certain of the lipids. Irving's (5, 7) histological findings indicate that the lipids extracted before decalcification were mainly intracellular, whereas extracellular matrix-bound lipids were extracted afterward. The lipid composition of extracts made before demineralization support this in that it was similar to that of soft tissues.

Regarding the bearing of these findings on the nature of the sudanophilic material at sites of new calcification, it seems unlikely that one lipid was solely responsible for the observed stain; but the fact that the lipid in extracts made after demineralization was rich in acidic lipids and poor in neutral phospholipids suggests that the material responsible for the Sudan black stain was primarily composed of acidic lipids.

The author expresses his appreciation to Mr. P. C. King for valuable technical assistance. The author is a Career Development Awardee, U.S. Department of Health, Education and Welfare.

This work was supported by U. S. Public Health Grant No. DE-00876, National Institute for Dental Research.

Manuscript received 8 May 1967; accepted 11 September 1967.

References

1. Wells, H. G. 1914. Chemical Pathology. Saunders, Philadelphia. 2nd edition. 402.
2. Irving, J. T. 1958. *Nature*. **181:** 704.
3. Irving, J. T. 1959. *Arch. Oral Biol.* **1:** 89.
4. Irving, J. T. 1960. *Clin. Orthopaed.* **17:** 92.
5. Irving, J. T. 1963. *Arch. Oral Biol.* **8:** 735.
6. Irving, J. T. 1963. *Arch. Oral Biol.* **8:** 773.
7. Irving, J. T. 1965. *In* Calcified Tissues. L. J. Richelle and M. J. Dallemagne, editors. Les Congrès et Colloques de l'Université de Liège, Liège. 313.
8. Irving, J. T., and R. E. Wuthier. 1961. *Arch. Oral Biol.* **5:** 323.
9. Irving, J. T. 1965. *Arch. Oral Biol.* **10:** 189.
10. Enlow, D. H., and J. L. Conklin. 1964. *Anat. Rec.* **148:** 279.
11. Enlow, D. H., J. L. Conklin, and S. Bang. 1965. *Clin. Orthopaed.* **38:** 157.
12. Leach, A. A. 1958. *Biochem. J.* **69:** 429.
13. Zambotti, V., I. Cescon, B. Bonferroni, and L. Bolognani. 1962. *Experientia*. **18:** 318.
14. Howell, D. S., J. F. Marquez, and J. C. Pita. 1965. *Arth. and Rheum.* **8:** 1039.
15. Cruess, R. L., and I. Clark. 1965. *Biochem. J.* **96:** 262.
16. Dirksen, T. R. 1963. *J. Dent. Res.* **42:** 128.
17. Dirksen, T. R., and K. G. Ikels. 1964. *J. Dent. Res.* **43:** 246.
18. Shapiro, I. M., R. E. Wuthier, and J. T. Irving. 1966. *Arch. Oral Biol.* **11:** 501.
19. Shapiro, I. M., and R. E. Wuthier. 1966. *Arch. Oral Biol.* **11:** 513.
20. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
21. Folch, J. 1952. *In* Phosphorus Metabolism. W. D. McElroy and B. Glass, editors. John Hopkins Press, Baltimore. **3:** 186.
22. Wuthier, R. E. 1966. *J. Lipid Res.* **7:** 558.
23. Wuthier, R. E. 1966. *J. Lipid Res.* **7:** 544.
24. Rouser, G., G. Kritchevsky, D. Heller, and E. Lieber. 1963. *J. Am. Oil Chemists' Soc.* **40:** 425.
25. Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller, and J. S. O'Brien, 1961. *J. Am. Oil Chemists' Soc.* **38:** 544.
26. Müldner, H. G., J. R. Wherrett, and J. N. Cumings. 1962. *J. Neurochem.* **9:** 607.
27. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. *Biochem. J.* **90:** 374.
28. Maruo, B., and A. A. Benson. 1959. *J. Biol. Chem.* **234:** 254.
29. Dawson, R. M. C. 1960. *Biochem. J.* **75:** 45.
30. Dawson, R. M. C., N. Hemington, and J. B. Davenport. 1962. *Biochem. J.* **84:** 497.
31. Burstone, M. D. 1957. *Arch. Path.* **63:** 164.
32. Entenman, C. 1961. *J. Am. Oil Chemists' Soc.* **38:** 534.
33. Dittmer, J. C., and R. M. C. Dawson. 1961. *Biochem. J.* **81:** 535.
34. LeBaron, F. N., and E. E. Rothleder. 1960. *In* Biochemistry of Lipids. G. Popják, editor. Pergamon Press, London. 1–7.
35. DiSalvo, J., and M. Schubert. 1967. *J. Biol. Chem.* **242:** 705.
36. Abramson, M. B., R. Katzman, and H. P. Gregor. 1964. *J. Biol. Chem.* **239:** 70.
37. Abramson, M. B., R. Katzman, C. E. Wilson, and H. P. Gregor. 1964. *J. Biol. Chem.* **239:** 4066.
38. Nash, H. A., and J. M. Tobias. 1964. *Proc. Nat. Acad. Sci. U. S.* **51:** 476.
39. Rojas, E., and J. M. Tobias. 1965. *Biochim. Biophys. Acta.* **94:** 394.
40. Hendrickson, H. S., and J. G. Fullington. 1965. *Biochemistry*. **4:** 1599.
41. Termine, J. D., R. E. Wuthier, and A. S. Posner. 1967. *Proc. Soc. Exptl. Biol. Med.* **125:** 4.