Lipid Metabolism in Normal and Rachitic Rat Epiphyseal Cartilage* (34092)

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(Introduced by F. J. Stare)

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The importance of lipid metabolism as related to calcification has received impetus by recent studies. While protein-polysaccharides have been implicated increasingly in the process of calcification (1), Irving has made pertinent histochemical observations noting the presence of a lipopolysaccharide at the calcification front in cartilage (2, 3). Howell and Carlson believe that these lipopolysaccharides are sulfated, and further studies by Howell have shown a striking difference in phospholipid profiles from rachitic and normal animal cartilage (4, 5). It has been postulated that vitamin D deficiency may cause changes in calcium translocation by affecting the selectivity of cation transfer (*i.e.*, Ca2+) through membrane coated with phospholipid.

Calcium binding has been shown to occur by a phospholipid protein complex (6). A regulatory function for lipids in cell membrane transport of electrolytes and other tissues has been extensively studied and a possible selective role for these compounds in mineralization or water transfer at calcifying sites has been theorized (7).

On this basis it appeared worthwhile to study lipid metabolism in weanling rat epiphyseal cartilage, a structure which is rapidly undergoing sequential changes in calcification and which, would, therefore, lend itself to such a study.

Methods and Materials. Animals and tissue preparation. Male weanling rats, 21 days old (40–45 g) of the Sprague-Dawley strain (Charles River Laboratory) were fed on Purina chow and water *ad libitum* until experimentation. The rachitic rats were fed a rachitogenic diet (Rachitogenic Diet No. 2, Nutritional Biochemicals Co.) from weaning (21 days old) until experimentation usually 1 or 2 weeks. The animals were sacrificed by decapitation and the lower extremities were rapidly dissected free. The proximal tibial and distal femoral epiphyses after exposure were separated from their shafts by a slice through the metaphyseal junction of the epiphyseal plate. The epiphyses were further cleaned of adherent tissue and 3 or 4 longitudinal slices were made approximately 0.5–1.0 mm in thickness. The slices were kept on an iced petri dish and finally placed in tared 25-cc incubation media and fitted with a center glass well. Approximately 250–300 mg of sliced cartilage was incubated in each flask. All incubations were begun less than 10–15 min after the animals were killed and 4–6 animals were used in each experimental group.

Media and Incubation. The incubation media used in all experiments was Krebs-Ringer bicarbonate buffered at pH 7.4 which contained one-half the suggested calcium and magnesium concentration and fortified with glucose at a concentration of 100 mg/100 ml (0.11 *M*). 1-^{14}C-Labeled palmitic acid (sp act 2.5 mCi/mmole, New England Nuclear Co.) was dissolved in buffer containing 1% albumin and 10 μmoles of carrier palmitic acid was added and homogenized. Purity of the C^{14} palmitric acid was investigated by T.L.C. (thin-layer chromatography). The final concentration of radioactive 1-^{14}C palmitic acid added to each flask was 0.2 μmole

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or 0.5 μCi. After the cartilage was added to the tared flasks the wet weight of the cartilage segments was determined. The flasks were then covered with serum stoppers and incubated at 37° in a metabolic shaker (70–75 oscillations/min) following 5 min of aeration with a 95% O_2, 5% CO_2 mixture for 1–2 hr depending on the experimental conditions.

At the end of the incubation period, the flasks were immersed in ice and 0.3 cc of hyamine was injected into the center well of the flask and media CO_2 was liberated by the addition of 0.3 cc of 1 *N* sulfuric acid followed by incubation at 37° in a metabolic shaker (70–75 oscillations/min) for 30–45 min. The tissue was transferred to a centrifuge tube and washed three times with cold Krebs-Ringer bicarbonate solution until the washings contained no radioactivity, after which the tissue was homogenized at 4° for 10 min in a Virtis 45-homogenizer at high speed with 20 ml of methanol: chloroform (1:2 v/v). An extraction of lipids from previously decalcified cartilage gave similar results as related to the incorporation of radioactivity into various lipid fractions. This type of extraction reduced the total uptake of the label into the palmitic acid and the free fatty acid fractions only.

Extraction and analysis of lipids. Total lipids were extracted by the Folch procedure with three extractions of methanol: chloroform (1:2 v/v) (8). The extract containing the lipids was evaporated to dryness under a stream of nitrogen at 40° and the residue was dissolved in 1.0 cc of petroleum ether. The total lipid was then determined from a dry weight measurement in preweighed test tube, redissolved in methanol:chloroform (1:2 v/v), and washed according to Folch. Aliquots of the total lipid-soluble products were assayed for radioactivity and analyzed by chemical and thin-layer chromotography for further characterization. Ascending thin-layer chromatography of the lipids was carried out on glass plates coated with silica gel-G as described by Stahl (9). Neutral lipid chromatography was performed by the method of Closter and Fletcher using a solvent system of petroleum ether:diethyl ether:acetic acid (85:15:2 v/v), which caused no migration of the phospholipid fractions (10).

Quantitative determination of the various lipids was then carried out. Esterified fatty acids were determined according to Stern and Sapiro (11); free fatty acids by the method of Itaya and Ui (12); cholesterol esters according to Brown *et al.* (13); and phospholipid phosphorus according to Bartlett (14). Glyceride-glycerol was determined according to Lambert and Neish (15). Spots identified in thin-layer chromatograms were isolated and eluted from the gel with petroleum ether or methanol and dissolved in 10 cc of counting solution (0.4% PPO; 0.005% POPOP per liter of toluene). Identified spots from duplicate thin-layer chromatograms were extracted with methanol:chloroform (1:2 v/v) and quantitatively determined by the methods described. The recovery was 82–95%. The chemical determinations were carried out on the lipid extracts and on the individual zones eluted from the thin-layer chromatograms. Solutions were counted in a lipid scintillation system where the degree of quenching was estimated by channels ratio, and the data were corrected arithmetically. The measurement of $^{14}CO_2$ conversion from palmitic acid was performed as described by Snyder and Godfrey (16).

Results. Although other animal cartilaginous and osseous tissues have been studied as to their lipid content (4, 17–19), rat epiphyseal cartilage has not, to our knowledge, been investigated. In Table I the values for DNA and RNA determinations on the normal control and rachitic cartilage are given. It can be observed (Table II) that there was a significant amount of lipid in rat

TABLE I

	DNA (mg/100 mg dry wt ± SD)	*p*	RNA / DNA
Control (18)	0.129 ± 0.028	<0.01	1.14
Rachitic (21)	0.167 ± 0.031		1.24
	RNA (mg/100 mg dry wt ± SD)		
Control (18)	0.148 ± 0.027	<0.01	
Rachitic (21)	0.208 ± 0.048		

TABLE II. Rat Epiphyseal Cartilage from Normal and Rachitic 28-Day-Old Animals was Isolated and Incubated in Individual Flasks Containing 3.0 cc of Incubation Media and 1-^{14}C Palmitic Acid as Described at 37°.[a]

	Total lipids	
	mg lipids/mg DNA	mg lipid/100 mg dry weight
Control	63.16 ± 3.85 (14) (1.029)	8.15 ± 0.51 (0.13)
Rachitic	72.23 ± 5.51 (10) (2.24)	12.08 ± 2.26 (0.56)
p	<0.01	<0.001

	mg lipid/mg DNA			
	Triglyceride[b]	Phospholipid[c]	Free fatty acid	Total cholesterol
Control	54.1 ± 3.05 (0.82)	6.9 ± 1.3 (0.34)	0.88 ± 0.06 (0.0016)	0.131 ± 0.033 (0.088)
Rachitic	54.7 ± 6.7 (2.11)	13.4 ± 1.5 (0.47)	1.13 ± 0.8 (0.0560)	2.80 ± 0.38 (0.119)
	NS	<0.001	<0.005	<0.001
	% of total lipids			
Control	85.65	10.92	1.4	0.20
Rachitic	75.7	18.53	1.6	3.87

[a] The data represents the mean plus one SD observed in 24 consecutive experiments containing five flasks in each group. Values in parentheses are the SE. The dry weight of the normal rat cartilage used was 34.5% of the wet weight and the rachitic rat cartilage dry weight 25.3% of the wet weight.

[b] Calculated as tripalmitine.

[c] Calculated as phosphatidylcholine.

epiphyseal cartilage (8.15 mg/100 mg dry cartilage weight) which increased rapidly with the induction of exerimental low phosphate rickets. Accompanying this increase in total lipids in the rachitic cartilage was an increase in the phospholipid, free fatty acid, and cholesterol fractions. It has been reported that it is difficult to extract lipid from compact bone (19) and this observation was confirmed by us since the blender (Virtis 45) used could not effectively homogenize calcified cartilage in normal animals over 42 days old. The cartilage from the rats placed on a rachitogenic diet, however, showed at 28–32 days an increased zone of provisional calcification histologically indicative of early rickets. In extraction procedures utilizing cartilage decalcified with EDTA the lipid fractions were found in the matrix in similar amounts to those results obtained by extracting the calcified cartilage by Virtis homogenization.

In the incubation experiments utilizing 1-^{14}C palmitic acid (Table III) it can be seen that very little of this label was converted into CO_2 (approximately 3–4% in controls and about 1% in rachitic cartilage) because the glucose present in the media was used preferentially as the energy source (21).

While there was greater labeling of CO_2 in rachitic cartilage, it was proportionally less because of the great increase in total uptake of 1-^{14}C palmitic acid by the tissue. The large amount of labeled nonesterified fatty acid in both rachitic and normal epiphyseal cartilage probably is accounted for by a process of diffusion into the intercellular fluid and/or tissue since the tissue was washed and extracted thoroughly after incubation. It can be observed that the synthesis of triglyceride and phospholipid is linear with time (Fig. 1) and compares favorably to the synthesis of protein polysaccharide and collagen by this tissue (19).

The striking difference in the metabolism of 1-^{14}C palmitic acid by rat epiphyseal cartilage is again designated in Table III where the synthesis of triglyceride and phospholipid

TABLE III. The Incorporation of 1-^{14}C Palmitic Acid into the Cartilage Various Lipid Fractions and CO_2 Is Expressed as DPM mg Cartilage DNA × 10^3. For details of the incubation see text.

Time (min.)	$C^{14}O_2$	Total uptake of palmitic acid-1-^{14}C into tissue	Free fatty acid	Triglycerides	Phospholipid	Cholesterol and esters	% Recovery
			Control				
30	69.0	1467.0	715.0	208.0	105.0	71.0	79.6
60	90.0	2460.0	1635.0	341.0	239.0	109.0	98.2
90	103.0	2901.0	1875.0	558.0	374.0	182.0	106.8
			Rachitic				
30	—	—	—	—	—	—	—
60	84.0	10930	9137	772	709	331	100.9
90	173.0	11432	8521	1575	1055	310	101.8

in rachitic cartilage is increased fourfold over controls.

Discussion. These studies utilizing rat epiphyseal cartilage indicate that this tissue actively incorporated 1-^{14}C palmitic acid into lipid. It is apparent that rat epiphyseal cartilage is far richer than bone in lipid content since Leach found only 0.0629% total lipid in dry ox femurs (19). In the present studies the total lipid was 8.15% of the cartilage dry weight. In a previous study, Guri and Bernstein reported on the uptake of uniformly labeled ^{14}C glucose into lipid as well as other cartilaginous fractions noting that there was a proportionally greater uptake of ^{14}C glucose into the lipid fractions of the metaphysis and secondary ossification centers of rat epiphyseal cartilage (20,21). These changes were thought to be due to the greater cellular changes observed in these areas. In the present experiments, no attempt was made to separate the cartilage into the various anatomical areas and note should be made by the heterogeneity of this tissue.

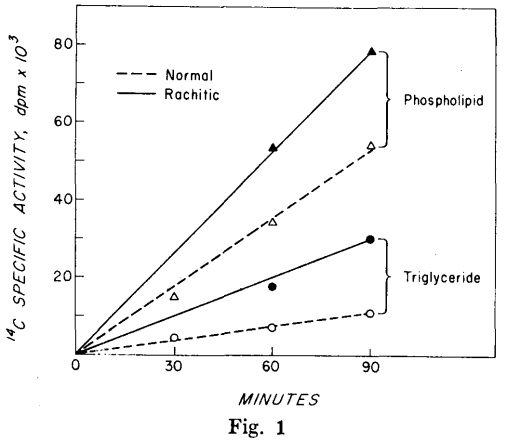

Fig. 1

In the incubation experiments described there was a significant uptake of the labeled substrate into triglycerides and phospholipids. These results are in accordance with those of Peck who found, when mouse calvaria were incubated with 1-3-^{14}C glycerol or inorganic ^{32}P, that most of the radioactive label could be found in lecithin and triglycerides.

In the only other study of the lipids contained in rachitic tissue, Howell *et al.* found that chloroform:methanol extracts of rachitic and normal costochondral junctions had quite different quantities of phospholipids with a reduction in the total phospholipid of rachitic tissue (4). Howell discounted the effect of diet on these parameters and it is unlikely that diet had an effect of diet on these parameters and it is unlikely that diet had an effect on our studies since the rachitic animals had a satisfactory weight gain (although their gross weight was less than in the controls as expected). However, our observations differ greatly from those of Howell in that we noted a fourfold increase in total phospholipid content in the rachitic rat cartilage. Howell further stated that there was a similar decrease in polysaccharide content of calf rachitic costochondral junctions but we

have noted an increase.[2] The reasons for these discrepancies is not immediately obvious, but it is well known that rachitic osteoid tissue is rich in protein-polysaccharide as shown histochemically (22, 23).

The increase in incorporation of 1-^{14}C palmitic acid into triglycerides and phospholipids and the total amount of triglyceride and phospholipid found in rachitic rat epiphyseal cartilage could have several explanations. The factors which initiate calcification are still undefined, but it may be that phospholipids serve as cation (*i.e.*, Ca2+) transfer agents to areas of the calcification front. The fact that the zone of provisional calcification in cartilage is rich (histochemically) in lipid serves to strengthen this hypothesis (2, 3, 24). Once calcification is initiated, the lipid content of the calcified tissue is reduced (17). While implying that phospholipids may be an important factor in calcification, it is also possible that vitamin D may have an effect on lipid synthesis, perhaps in activating an enzyme, such as a phospholipase, which would break down phospholipids. A reduced activity of phospholipases as well as lipases could account for the findings of large amounts of lipid in rachitic cartilage. As well, vitamin D has been shown to effect kidney mitochondria, which are rich in phospholipids, as regards calcium trnaslocation (25).

It is evident that further study of lipids and their relationship to calcification is needed and should prove rewarding in the understanding of biological calcification.

Summary. Lipid synthesis in rat epiphyseal cartilage has been studied in relation to normal growth and the production of rickets. Cartilage was incubated *in vitro* in modified Krebs-Ringer bicarbonate buffer with added 1-^{14}C palmitic acid. In normal 28-day-old male weanling rats, the total uptake of 1-^{14}C palmitic acid from the media showed a gradual increase with time. Fractionation of the lipids showed 64.6% of the label in free fatty acids; 19.2% in triglycerides; 12.8% in phospholipids; and 6.2% in cholesterol and cholesterol esters. In rachitic rats of comparable age, the total uptake of 1-^{14}C palmitic acid was increased significantly, indicating an increased rate of lipid synthesis.

[2] Unpublished observations.

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