The Effect of Vitamin D Deficiency on the Lipids of Bone Matrix[1] (35277)

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Recent work has shown that significant amounts of lipids are present in the matrix of rat bone (1, 2) and it has been demonstrated that hypervitaminosis D increases all lipid components with the major increase being in the phospholipid fraction (3). There has been speculation that vitamin D exerted its effect on skeletal metabolism through alterations in phospholipid metabolism (4, 5). Recently, it has been shown that in phosphate-deficient rickets there was an increase in lipid synthesis (6). The following series of experiments were carried out in order to assess the effect of a true vitamin D deficient state, *i.e.*, diet containing optimal amounts of calcium and phosphate upon the lipids of rat bone.

Material and Methods. Weanling male rats of the Holtzman strain weighing 50 g were fed a vitamin D-free semisynthetic diet which contained 60% cerelose, 24% vitamin-free casein, 2% alphacel, 10% Mazola oil, 4% salt mix, and adequate amounts of all known vitamins except vitamin D. The diet contained (%): magnesium, 0.05; sodium, 0.12; potassium, 0.36; calcium, 0.8; and phosphate, 0.4. After 4 weeks on this diet, at which time they weighed approximately 90 g, the control rats received 75 IU of vitamin D_2 (Calciferol[2]) in sesame oil three times a week and the D-deficient rats received only sesame oil. After 60 days on this regimen, 10 rats from each group were decapitated and their humeri, femora, and tibiae removed and cleaned of soft tissues. The epiphyses were pried off from the metaphyses which were then separated from the diaphyses by transection with a saw under a cold saline drip. After the marrow was removed by washing with a spray of ice-cold saline, all epiphyses, metaphyses, and disphyses of a single rat were pooled and the bones were frozen, lyophilized, and ground in a Spex Grinder[3] in the cold for 2 min. The lipids were extracted and washed according to the method of Folch *et al.* (7). The total lipids were determined gravimetrically, the lipid phosphorus was measured according to the method of Marinetti (8), and the phospholipid was calculated by multiplying the phosphorus content of the lipid extract by 25 (9). The triglycerides were determined by the method of Van Handel *et al.* (10), the total fatty acids were titrated according to the method of Albrink (11), and the total cholesterol was determined by a modification of the Leiberman-Burchard reaction (12). The ash weight was determined after ashing a sample of dried defatted bone powder in a muffle furnace at 680° for 48 hr and the organic fraction was calculated by subtracting the percentage of ash from 100. All lipid values were expressed as milligrams per gram of organic weight except fatty acids which were expressed as milliequivalents per gram organic. These methods have been previously used and reported (1). The powdered epiphyses, metaphyses, and diaphyses which were left following extraction of lipids and determination of the ash weight were pooled, separately decalcified with EDTA, extracted with chloroform–methanol, and reextracted with acidic chloroform–methanol as outlined by Wuthier (2). Duplicate samples of the original and subsequent extracts were applied to silicic acid thin-layer chromatograms and separated ac-

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[2] Roxan and Phillips.

[3] Spex Industries, Scotch Plains, New Jersey.

TABLE I. Body Weight, Serum Calcium, and Serum Phosphorus.[a]

	Wt (mg) at sacrifice	Serum (mg/100 ml)	
		Calcium	Phosphorus
Control	326 ± 6.4 (10)	10.7 ± 0.17 (10)	7.9 ± 0.12 (10)
D deficient	203 ± 7.5[b] (10)	6.8 ± 0.16[b] (10)	13.1 ± 0.17[b] (10)

[a] ± standard error; number of animals is given in parentheses.
[b] $p < 0.01$.

cording to the method of Wagner *et al.* (13). Identification and quantification were carried out by the method of Blecher and Abramson (14).

Serum calcium determinations were carried out using atomic absorption spectrophotometry; and serum phosphorus was determined by the method of King (15).

Results. The decrease in body weight gain, the lowered serum Ca and elevated serum P of the vitamin D-deprived rats (Tables I and II) and histologic examination clearly show evidence of true rickets in rats fed a diet containing a normal calcium to phosphorus ratio as noted by DeLuca *et al.* (16). There was an increase in the amount of organic matter present in all regions of the bone with the greatest change being seen in the metaphyseal region (Table II).

The lipids extracted before decalcification are described in Table III. Vitamin D deficiency increased the total lipids of the epiphysis and diaphysis but decreased them in the metaphyseal region. There was no significant alteration in the phospholipid content of any of the regions. In the deficiency state, cholesterol was increased in the diaphyseal region but unchanged in the other areas, total fatty acids were increased in both the epiphysis and diaphysis but decreased in the metaphysis, and triglycerides were increased in the epiphysis and diaphysis and decreased in the metaphyseal region. It is to be noted that the total lipid is less than the sum of the parts because the fatty acid value is derived from fatty acids of triglycerides, phospholipids, cholesterol esters, and free fatty acids.

TABLE II. Organic Fraction (%) of Control and Treated Animals.[a]

	Control	D deficient
Epiphysis	48.1 ± 1.1 (10)	56.6 ± 0.8 (10)[b]
Metaphysis	34.7 ± 0.4 (10)	44.1 ± 0.7 (10)[b]
Diaphysis	29.1 ± 0.4 (10)	31.6 ± 0.3 (10)[b]

[a] ± standard error; number of animals is given in parentheses.
[b] $p < 0.01$.

The percentage of the total extracted phospholipids are shown in Table IV. Statistical differences were not possible because of insufficient material even with pooled samples. As shown, there are no striking differences in the phospholipids extracted from the bones of D-deficient animals compared to controls.

Discussion. The alterations in serum calcium and phosphorus of calcium and phosphate demonstrate that a true D-deficient state using a diet with optimal amounts of calcium and phosphorus was produced. An increase in osteoid has been long recognized in bones of D-deficient animals. However, the accumulation of osteoid is greatest in the metaphysis and least in the diaphysis. In these studies there is an increase in the total lipid in the epiphyseal region with a decrease in the metaphysis and diaphysis. Thus in regions where osteoid is accumulating, the chemical content of osteoid is altered with the decrease being seen in the metaphysis. The total lipid changes are apparently due primarily to alterations in triglyceride and fatty acids as these values most closely parallel the total lipid value. There is thus essentially no change in the phospholipid or cholesterol of the epiphysis or metaphysis although the diaphyseal values do show significant changes. Havivi and Bernstein (6) have reported an increase in phospholipids, free fatty acids, and total cholesterol in epiphyseal cartilage in phosphate-deficient rickets when these values are calculated per milligram of DNA as well as percentage of

TABLE III. Lipid Values of Lipids Extracted Before Decalcification.[a]

		Control	D deficient
Total lipids (mg/g organic)	Epiphysis	83.9 ± 6.1 (10)	120 ±10[b] (10)
	Metaphysis	71.1 ± 7.2 (9)	51.2 ± 4.6[b] (10)
	Diaphysis	7.62 ± 0.92 (10)	16.4 ± 1.4[b] (10)
Phospholipids (mg/g organic)	Epiphysis	7.90 ± 0.44 (10)	8.66 ± 0.32 (10)
	Metaphysis	9.49 ± 0.69 (10)	9.75 ± 0.59 (10)
	Diaphysis	1.56 ± 0.14 (10)	2.38 ± 0.16[b] (10)
Cholesterol (mg/g organic)	Epiphysis	3.13 ± 0.21 (9)	3.14 ± 0.20 (9)
	Metaphysis	2.93 ± 0.25 (9)	3.25 ± 0.36 (9)
	Diaphysis	0.484 ± 0.048 (8)	0.917 ± 0.100[b] (9)
Total fatty acid (mg/g organic)	Epiphysis	22.41 ± 2.67 (10)	24.93 ± 2.56 (9)
	Metaphysis	17.19 ± 2.68 (9)	9.84 ± 0.77[c] (10)
	Diaphysis	1.77 ± 0.30 (9)	2.68 ± 0.27[c] (10)
Triglyceride (mg/g organic)	Epiphysis	2.33 ± 0.10 (9)	2.77 ± 0.21 (9)
	Metaphysis	1.57 ± 0.16 (8)	1.06 ± 0.12[c] (10)
	Diaphysis	0.181 ± 0.020 (8)	0.676 ± 0.073[b] (10)

[a] ± standard error; number of animals is given in parentheses.
[b] $p < 0.01$.
[c] $p < 0.05$.

the total lipids. They also reported an increase in DNA and RNA per 100 mg of dry weight. No details were given concerning the determinations of DNA and RNA in bone and this is important as quantitative recoveries of DNA and RNA from small amounts of mineralized tissue is very difficult. Also, the dry weight of the control bones of their series was greater than that of the rachitic ones, which is inevitable since the control bones contain significantly more mineral than the rachitic bones. Hence the validity of expression of DNA per mg of dry weight or lipids per mg of dry weight is open to question. Also these authors found increased rates of synthesis in their phospholipid and triglyceride fraction. These differences may perhaps be explained by their studying phosphate rickets, *i.e.*, phosphate deficiency while in the present study true rickets was examined.

The phospholipids extracted from normal rat bone are similar to those reported previously by Cruess and Clark (3) with the exception that small amounts of phosphatidyl serine were extractable prior to decalcification. Wutherier (2) also extracted small amounts of phosphatidyl serine before decalcification but larger amounts were found following decalcification. However, he was dealing with fetal bone of calves rather than the more mature bone of rats and it may be that this accounts for the differences noted. There were no significant changes seen in the different phospholipids between the D-deficient and control bones. However, statistical analyses of these samples were not possible due to limitation in material.

There has been speculation that vitamin D exerted its influence on calcium metabolism by altering phospholipid metabolism. Thus Hoyosha (5) found an increased incorporation of ^{32}P into mitochondria of kidney and gut but not of liver. Cruess and Clark reported a relative increase in the phospholipids in hypervitaminosis D (1). Irving (4) had noted a sudanophilic material which was absent in the rachitic state and returned on treatment with vitamin D and felt that this represented phospholipid. An adequate D-deficient state was obtained in the present experiment and no gross differences in either the total extractable phospholipid or the relative proportions of phospholipids have been found. It is therefore considered unlikely that vitamin D exerts its effect on bone by a direct effect on phospholipid metabolism.

Howell *et al.* (17) compared rachitic costo-

TABLE IV. Phospholipid Values Before and After Decalcification.[a]

Extract:	First (Chloroform/methanol)				Second (Chloroform/methanol)				Third (Acidic chloroform/methanol)			
	Before decalcification				After decalcification							
	Control		D-Def.		Control		D-Def.		Control		D-Def.	
	Total (mg/g organic)	% of total lipid P	Total (mg/g organic)	% of total lipid P	Total (mg/g organic)	% of total lipid P	Total (mg/g organic)	% of total lipid P	Total (mg/g organic)	% of total lipid P	Total (mg/g organic)	% of total lipid P
D	1.56	LL 11.1	2.38	LL 7.6	0.20	LL 17.0	0.31	LL 22.2	1.15	LL 35.4	1.75	LL 30.0
		SM 22.4		SM 17.5		SM 29.6		SM 28.8		SM 22.5		SM 28.1
		L 51.0		L 58.8		L 32.7		L 28.8		L 21.7		L 26.4
		PS 8.3		PS 5.4		PS 14.4		PS 20.2		PS 15.0		PS 7.2
		PE 7.2		PE 10.7		PE 6.3		PE 0		PE 5.4		PE 8.3
M	9.49	LL 15.5	9.75	LL 7.6	0.38	LL 17.8	0.34	LL 13.0	1.34	LL 24.8	1.34	LL 15.8
		SM 17.2		SM 16.3		SM 23.5		SM 12.7		SM 47.0		SM 28.7
		L 41.3		L 59.8		L 37.8		L 29.6		L 9.4		L 26.2
		PS 12.8		PS 6.3		PS 12.2		PS 20.4		PS 13.7		PS 17.7
		PE 13.2		PE 10.0		PE 8.7		PE 24.3		PE 5.1		PE 11.6
E	7.9	LL 13.8	8.66	LL 8.0	0.36	LL 6.9	0.62	LL 13.9	0.91	LL 11.4	1.63	LL 36.4
		SM 20.6		SM 27.6		SM 32.4		SM 30.4		SM 36.5		SM 25.0
		L 45.4		L 45.4		L 29.7		L 49.1		L 29.7		L 19.1
		PS 10.3		PS 11.3		PS 14.1		PS 3.3		PS 15.6		PS 10.3
		PE 9.9		PE 7.7		PE 16.9		PE 3.3		PE 6.8		PE 9.2

[a] LL = lycolecithin; SM = sphingomyelin; L = lecithin; PS = phosphotidyl serine; PE = phosphotidyl ethanolamine.

chondral junctions with controls and noted a decrease in the phospholipid of the rachitic cartilage and an alteration of the pattern of phospholipids. However, the tissue studied was different, the animals were evidently quite ill, and the methods used were quite different; and the present study did not confirm this earlier work.

There has been much indirect evidence that phospholipids may play a role in calcification. Johnson (18) proposed a scheme whereby there was an orderly progression from neutral fat to phospholipid which occurred just prior to calcification. An analysis of the matrix of the D-deficient bones would tend to suggest that neutral fats are formed in normal or increased amounts but that conversion to phospholipid is perhaps arrested. It may be that the failure of rachitic osteoid to calcify is in part the result of this accumulation of neutral fat. Wuthier (2) suggested that phosphatidyl serine was bound to the calcium of mineralizing tissues because it was more easily extractable following decalcification. Our data neither confirms nor contradicts this hypothesis but there does not appear to be an accumulation of phosphatidyl serine in the undermineralized rachitic osteoid.

Summary. The bones of control and vitamin D-deficient animals have been analyzed to determine whether the D-deficient state affects the lipids extractable from these bones. A decrease in serum calcium and an increase in serum phosphorus was noted and all D-deficient animals had an increase in the percentage of the organic fraction of the bones. There was an increase in the total lipids extractable from the epiphysis, metaphysis, and diaphysis; and a significant increase was found in the diaphyseal phospholipids and cholesterol. Significant decrease was noted in the metaphyseal fatty acid and an increase in the diaphyseal fatty acid. There was a decrease in the metaphyseal triglyceride and an increase in the diaphyseal triglyceride. It is concluded that perhaps there is an arrest in the conversion of neutral fats to phospholipids but that vitamin D-deficiency does not have a primary effect on phospholipid metabolism.

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