Alterations in the Lipids of Bone caused by Hypervitaminosis A and D

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1. The total lipid, phospholipid, total and free fatty acid, free and esterified cholesterol contents of the long bones of normal, hypervitaminotic A, D and A plus D rats were determined. 2. Toxic amounts of vitamin A decreased the total fatty content, whereas toxic amounts of vitamin D increased triglycerides, esterified cholesterol and in particular the phospholipids of bone. 3. An interaction occurred between toxic amounts of vitamins A and D, which prevented, to a large extent, the alterations in bone lipids that occur in hypervitaminosis D. 4. The studies suggest an involvement of vitamin D in lipid metabolism and tend to support the idea that lipids are involved in ossification.

Although there is considerable information about the collagen, mucopolysaccharide and mineral composition of bone (Bourne, 1956), very little is known about the lipids. Recent histochemical studies suggest that lipids may be involved in calcification (Johnson, 1960) and ossification (Irving, 1958). The following studies were undertaken to characterize the lipids of bones of normal rats and of rats given vitamins A and D, in quantities sufficient to alter other constituents of bone (Clark & Smith, 1964), in an attempt to ascertain whether lipids are involved in osteogenesis. A preliminary account of some of this work has been reported (Cruess & Clark, 1964).

METHODS

Albino male rats of the Holtzman strain, weighing about 150g., were fed on a complete synthetic laboratory diet containing sufficient amounts of vitamins A and D for adequate growth and maintenance. The vitamin A palmitate was generously supplied by Merck, Sharpe and Dohme Research Laboratories and the vitamin D₂ (calciferol) by the Roxane-Philips Co. The supplemental vitamins were administered daily by stomach tube/100g. body wt. Vitamin A palmitate was diluted with purified sesame oil. The control rats (group I) received sesame oil; group II received 15000 i.u. of vitamin A; group III received 30000 i.u. of vitamin D; group IV received 15000 i.u. of vitamin A and 30000 i.u. of vitamin D. Fifteen animals in each group were maintained on the above regimens for 44 days, during which time three of the animals from group III and one from group IV had died. The animals were decapitated and the femora, tibiae, humeri, radii and ulnae were removed after subperiosteal dissection of soft tissues. The epiphyseal ends of the bones were prised off and the remaining cartilage was

trimmed by transection through the metaphyseal end of the bone close to the epiphyseal line, removing as little as possible of the metabolically active metaphysis. The marrow elements were removed by flushing the marrow cavity with a jet of distilled water through a 22-gauge hypodermic needle. The bones were frozen immediately with solid CO₂, freeze-dried at -50° and stored in a desiccator over P₂O₅ until used. The bones of each rat were pulverized in a mixer mill (model 5000; Spex Industries, New Jersey) for 2 min., and the lipids were extracted and purified by the method of Folch, Lees & Sloane-Stanley (1957). The washed extract was stored under nitrogen in the cold until used.

Total lipid content was determined gravimetrically after evaporation of the solvent. Free cholesterol was determined on the dry residue by a modification of the Liebermann-Burchard reaction (Shafel, 1944). Lipid phosphorus was determined on a 2 ml. sample of extract by the method of King (1956), and the phospholipid content was calculated by multiplying P by 25 (Wittcoff, 1951). The fatty acids and total cholesterol were determined in a 10 ml. sample of extract by the method of Albrink (1959), except that 5 mN-sodium methoxide was used in place of 4 mN-NaOH in the titration step. Free fatty acids were calculated by subtracting from the chemically determined total fatty acids the sum of the amounts found in phospholipids (with lecithin as a representative phospholipid), and in the triglycerides (with tristearin as a representative triglyceride), and in the cholesterol esters. The colorimetric procedure for total cholesterol was that of Abel, Levy, Brodie & Kendall (1952). The triglycerides were measured by the method of Van Handel, Zilversmit & Bowen (1957), except that the oxidation was stopped with sodium sulphite (0.5M).

Dried defatted weight was determined by drying 100 mg. of extracted bone in an oven at 110° for 24 hr. The ash content of the dried defatted bone was measured after ashing in a muffle furnace at 650° for 18 hr. The organic content was calculated by subtracting the ash weight from the dried defatted weight.

Table 1. *Changes in the body and bone weights of rats receiving dietary vitamin supplements for 44 days*

Experimental details are given in the text. Values significantly different from control values are indicated by: * $P < 0.001$; ** $P < 0.01$. Significant interaction between vitamins A and D is indicated by: † $P < 0.001$; †† $P < 0.025$.

	Control	Vitamin A	Vitamin D	Vitamins A and D
Initial weight (g.)	149	152	151	150
Final weight (g.)	306	182*	176*	177†
Ash (%)	68.3	69.6**	58.9*	64.6††
Organic fraction (%)	31.7	30.4	41.1*	35.4††

The experimental design was that of a 2×2 factorial with unequal replications per group and an analysis of co-variance was performed on the data according to the method of Rao (1952).

RESULTS

Although the animals that received vitamin A failed to gain weight normally, the ash content of their bones was slightly, though significantly, elevated (Table 1). Rats treated with vitamin D also failed to gain weight as well as the control animals, but there was a marked decrease in the ash content of the bones of these animals. Although the gains in body weight of the rats given the combination of excess of vitamins A and D were about the same as those of the animals given vitamin A or vitamin D, calculation of an analysis of co-variance indicates that an interaction exists between these vitamins and that the gain in body weight after the combined administration is significantly greater than could be expected if these vitamins acted independently. Moreover, as has been reported by Clark & Smith (1964), the deviations in the values of the ash and organic content of the bones of the rats given the combination of vitamins A and D from those of the controls were not as great as those of the rats given just vitamin D, indicating an interaction between these vitamins with respect to bone ash and organic content.

The lipid analyses of the bones are shown in Table 2. Hypervitaminosis A did not change significantly the lipid values of the bones with the exception of total fatty acids, and this fraction only if expressed as mg./g. of bone. Toxic amounts of vitamin D, however, increased significantly the total lipid content and the individual components of the lipids when expressed as mg./g. of bone and, with the exception of free fatty acids and free cholesterol, they increased other lipids when expressed as percentage of organic matter. The lipid values of bones from animals that received vitamin A together with vitamin D were greater than those of the control but not as elevated as those of the bones of the hypervitaminotic D rats. How-

ever, significant interactions between the vitamins only were found in the phospholipids and total fatty acids when expressed as mg./g. of bone.

An examination of the proportion of the lipids (Table 2) shows that only total fatty acids were affected by hypervitaminosis A. Excess of vitamin D increased the percentage of phospholipids from 16 to 24, and although this increase was not statistically significant it is considered that this is a true increase since only two of the treated animals had values within the normal range. The increase in total fatty acids results from the fatty acids derived from the increased phospholipids.

DISCUSSION

The only lipid values of normal bone found in the literature were those of Leach (1958), who reported values considerably lower than those reported above. However, since his methods of extraction and analysis and also the type of bone and species of animal were different from ours, the variation in results is not surprising.

It has been shown that in bones of hypervitaminotic D rats the proportion of mineral to organic matrix differs from that of control bones (Follis, 1950; Clark & Smith, 1964). Consequently, when the results are expressed as mg./g., conclusions about the composition of the matrix are misleading. If the lipid values are calculated as a fraction of the organic matter, of which they are an integral part, it is evident (Table 2) that vitamin D increased the absolute amount of phospholipids, triglycerides and total cholesterol, and of their constituent fatty acids. Further, it appears that vitamin D increases the phospholipid fraction to a greater extent than the other components, indicating an altered lipid composition (Table 2). Whether these increases result from increased synthesis or decreased breakdown of the lipids is not apparent from these experiments. The persistence of the phospholipids in the bones of the hypervitaminotic D rats may in some way be associated with the failure of calcification, since it

Table 2. Analyses of the bone lipids from rats receiving dietary vitamin supplements for 44 days

Experimental details are given in the text. Values significantly different from control values are indicated by: * $P < 0.001$; ** $P < 0.01$; *** $P < 0.005$. Significant interaction between vitamins A and D is indicated by: † $P < 0.001$; ‡ $P < 0.025$.

	Control			Vitamin A			Vitamin D			Vitamins A and D		
	(mg./g.)	(% of organic fraction)	(% of total lipid)	(mg./g.)	(% of organic fraction)	(% of total lipid)	(mg./g.)	(% of organic fraction)	(% of total lipid)	(mg./g.)	(% of organic fraction)	(% of total lipid)
Total lipids	9.60	2.96	—	11.47	3.77	—	18.80*	4.58*	—	16.63	4.77	—
Phospholipids	1.54	0.46	16.4	1.93	0.63	17.2	4.42*	1.07*	23.8	2.85††	0.81	18.4
Total fatty acids	4.21	1.39	44.3	4.09**	1.35	37.1**	9.71*	2.37*	53.3	6.75†	1.94	40.7
Free fatty acids	2.21	0.69	24.0	2.11	0.70	19.4	4.15***	1.07	25.1	2.73	0.78	18.5
Triglycerides	1.05	0.21	10.9	0.99	0.32	8.7	2.13*	0.52**	11.6	1.55	0.44	9.4
Total cholesterol	0.51	0.15	5.3	0.52	0.17	4.7	0.87*	0.22*	4.9	0.80	0.23	5.1
Free cholesterol	0.17	0.05	1.9	0.24	0.08	2.1	0.32*	0.08	1.7	0.27	0.08	1.9
Total lipid recovered (%)	—	—	56.6	—	—	50.0	—	—	65.4	—	—	51.4

has been observed that phospholipids disappear just before calcification (Johnson, 1960). Also, it has been shown that the incorporation of [^{32}P]orthophosphate into the intestinal mucosa of vitamin D-deficient rats is approximately one-third that of control animals, indicating a relationship between phospholipid metabolism and vitamin D (Thompson & DeLuca, 1964). Although a relationship appears to exist between vitamin D and phospholipid metabolism, it is impossible, at present, to state with certainty whether the phospholipids are intimately involved in ossification. However, the absence of lipid-staining material in the epiphyseal cartilage of rachitic animals and its return on treatment with vitamin D (Irving, 1959) suggest a relation between vitamin D, lipids and ossification.

Excess of vitamin A alone or in combination with excess of vitamin D tended to lower the total fatty acids, the major fraction of the lipids, of bone. Vitamin A also has been shown to decrease the collagen and mucopolysaccharide contents of bone (Clark & Smith, 1964).

The administration of excess of vitamin A to rats along with excess of vitamin D has been shown to prevent, to a large extent, the histological changes of hypervitaminosis D (Clark & Bassett, 1962) as well as the chemical changes (Clark & Smith, 1964). The interaction of the vitamins also occurs with many of the lipid fractions. The mechanism of action of vitamin A in preventing the accumulation of excess of matrix in hypervitaminosis D is still obscure. Vitamin A has been shown to hasten both osseous maturation and remodelling of normal bone (Wolbach, 1947). The excess of vitamin A may be acting in a similar fashion on this abnormal matrix, possibly by liberating cellular proteases (Fell, Mellanby & Pelc, 1956; Fell, Lucy & Dingle, 1961; Lucy, Dingle & Fell, 1961; Dingle, 1961) capable of hydrolysing the macromolecules of bone.

Only 50–65% of the chloroform-methanol-extractable material could be accounted for by the phospholipids, free fatty acids, triglycerides and total cholesterol (Table 2). The identification of the remaining 35–50% is under investigation.

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REFERENCES

- Abel, L. L., Levey, B. B., Brodie, B. B. & Kendall, R. E. (1952). *J. biol. Chem.* **195**, 357.
 Albrink, M. J. (1959). *Lipid Res.* **1**, 53.

- Bourne, G. H. (1956). In *The Biochemistry and Physiology of Bone*, p. 81. Ed. by Bourne, G. H. New York: Academic Press Inc.
- Clark, I. & Bassett, C. A. L. (1962). *J. exp. Med.* **115**, 147.
- Clark, I. & Smith, M. (1964). *J. biol. Chem.* **239**, 1266.
- Cruess, R. L. & Clark, I. (1964). *Fed. Proc.* **23**, 375.
- Dingle, J. T. (1961). *Biochem. J.* **79**, 509.
- Fell, H. B., Lucy, J. A. & Dingle, J. T. (1961). *Biochem. J.* **78**, 11 p.
- Fell, H. B., Mellanby, E. & Pelc, S. R. (1956). *J. Physiol.* **134**, 179.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Follis, R. H. (1950). In *2nd Conf. Metabolic Interrelations*, p. 221. Ed. by Reifenshtein, E. C., jun. New York: Josiah Macy, jun., Foundation.
- Irving, J. R. (1958). *Nature, Lond.*, **181**, 704.
- Irving, J. R. (1959). *Nature, Lond.*, **183**, 1734.
- Johnson, L. C. (1960). *Publ. Amer. Ass. Advanc. Sci.* no. 64: *Calcification in Biological Systems*, p. 117.
- King, E. J. (1956). *Microanalysis in Medical Biochemistry*, 3rd ed., p. 79. New York: Grune and Stratton.
- Leach, A. A. (1958). *Biochem. J.* **69**, 429.
- Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
- Rao, C. R. (1952). *Advanced Statistical Methods in Biometric Research*. New York: John Wiley and Sons Inc.
- Shaftel, A. G. (1944). *J. Lab. clin. Med.* **29**, 875.
- Thompson, V. W. & DeLuca, H. F. (1964). *J. biol. Chem.* **239**, 984.
- Van Handel, Z., Zilversmit, D. B. & Bowen, K. (1957). *J. Lab. clin. Med.* **50**, 152.
- Wittcoff, H. (1951). *The Phosphatides*, p. 162. New York: Reinhold Publishing Corp.
- Wolbach, S. B. (1947). *J. Bone Surg.* **29**, 171.