The Lipids of Ox Compact Bone

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Pikular (1955) determined the cholesterol content of compact bone after demineralization with hydrochloric acid. By the digitonide method, the cholesterol content of the organic matter (osseo-collagen) was found to be 0.0028–0.0040 g. of cholesterol/100 g. of dry ash-free protein. He also prepared procollagen by the method of Orekhovich, Tustanorskiĭ, Orekhovich & Plotinkora (1948) and showed cholesterol to be present, but did not measure the quantity. Pikular (1955) pointed out that the cholesterol content of the collagenous material was very similar to that found in myostroma by Nikol'skaya (1953) and concluded that the cholesterol was present in the form of loose and firm complexes with protein.

Spector (1956) records that compact bone from ox femur contains 0.1 g. of lipid/100 g. dry weight.

The present paper is a brief study of the lipids of undemineralized compact bone.

EXPERIMENTAL

Materials

Ox-femur compact-bone powder. The shafts from fresh ox femurs were freed from marrow, spongy bone, periosteum and traces of tendon at the point of attachment. The bones were washed quickly with acetone, a brush being used to clean the insides of the shafts. The latter and the points of attachment of the tendons were rasped to remove surface irregularities. A further superficial wash with acetone was given. It was considered that this procedure, whilst not removing significant amounts of compact-bone lipid, minimized contamination by bone marrow. The bones were broken into pieces measuring about 1 in. $\times$ $\frac{1}{2}$ in. $\times$ $\frac{1}{2}$ in. and ground to a fine powder in a Christy-Norris laboratory mill. The temperature of the powder from the mill did not rise above 30°. After mixing, one half was sieved. Any material not passing a 100 mesh British Standard sieve was reground and resieved.

A similar procedure was applied to a mixture of other ox-leg bones. This will be referred to as 'other bone material' in contrast with 'ox-femur compact bone'.

Methods

Sieving. Samples of the powders were sieved with British Standard sieves to obtain size distribution.

Moisture. Loss of weight on drying a thin layer of bone powder for 16 hr. at 105° was used to calculate moisture.

Extraction of lipid. A number of slightly different methods with Soxhlet extractors were employed on the ox-

femur compact-bone powder, whereas the other bone material was directly extracted with cold acetone. Soxhlet methods: (a) about 50 g. of bone powder was extracted with acetone, the powder was then reground and re-extracted (the material was so fine that regrounding only amounted to remixing); (b) as (a), two thimbles being used consecutively without change of flask or solvent; (c) as (b), but with CCl₄-ethanol (9:1, v/v); (d) about 50 g. of bone powder was extracted with acetone, the acetone was removed from the system and the bone residue was reground and extracted with ether. Some of the acetone-soluble material in the extraction flask was insoluble in the ether and was filtered off before the lipid was isolated. The yield from 50 g. of powdered bone was 30–40 mg. of lipid.

Cholesterol. Total and free cholesterol were determined by precipitating the digitonides by a scaled-up modification of the method of Schoenheimer & Sperry (1934) as modified by Sperry & Webb (1950), and finally by weighing the digitonide. The determinations were carried out on samples of lipids isolated during the determination of total lipid and therefore suffer from the disadvantage of possible variation in purity of the isolated material. The total cholesterol in the material extracted with cold acetone was determined after hydrolysis with ethanolic 2N-KOH for 2 hr. The weight of digitonide was multiplied by 0.25 to give cholesterol, and the difference between total and free cholesterol was multiplied by 1.73 to give the weight of cholesterol esters.

Phospholipids. The Fiske & Subbarow (1925) method, as modified by Yuen & Pollard (1951) for the determination of phosphate ions, was carried out on a solution obtained by digesting the lipid extracted by method (d) with HNO₃ in the presence of Ca(NO₃)₂, evaporating to dryness and dissolving the residue in water. The lipid P was multiplied by 25 to give the weight of phospholipid.

Fatty acids. The lipid was hydrolysed with ethanolic KOH; the ethanol was evaporated off, the residue suspended in 5% NaCl (minimizing the formation of emulsions) and extracted with CCl₄ to remove unsaponifiable matter. The solution was acidified and re-extracted with CCl₄ to obtain the fatty acids, which were then isolated. Triglycerides were calculated from the fatty acid content after allowing for cholesterol esters and phospholipids.

Glycerol. This was determined on the lipid extracted with cold acetone after hydrolysis by the method of Whyte (1946) adapted for one-tenth of the quantities described. There was insufficient material for the quantitative determination of glycerol on the Soxhlet-extracted material, as less bone powder was available for analysis, but qualitative indications were obtained for its presence.

Absorption spectra. Solutions (1.01 and 0.023%, both w/v) of the cold-acetone-extracted lipid in hexane (fraction from petroleum, special for spectroscopy, British Drug

Houses Ltd.) were used for the determination of the absorption spectra in the visible and ultraviolet regions respectively.

Examination of the residue. The residue obtained from the acetone-soluble lipid after re-extraction with ether [see extraction (d)] was examined on a microscale. After hydrolysis, it showed strong reaction with ninhydrin and gave a value of 1.5 g. of hydroxyproline/100 g. of organic material by the Neuman & Logan (1950) technique.

RESULTS

Size distribution of powdered compact bone. The results are expressed as percentage of original material, the sieve numbers being given first. On once-ground material: not passing 22, nil; 22–36, 1%; 36–44, 4%; 44–72, 25%; 72–100, 7%; 100–150, 19%; passing 150, 44%. On reground material: not passing 100, nil; 100–150, 40%; 150–200, 22%; passing 200, 38%.

Total lipid. Protein-containing material appeared in some of the extracts. The results for the various extractions are given in Table 1, and the following observations can be made: (1) The appearance of two phases in the extracted lipid, its incomplete solubility in ether and its darker colour coincided with the extraction of increased apparent percentages of lipid from the bone. However, where these effects were very small, or were eliminated by re-extraction of the lipid, there was good agreement between the different methods. (2) The presence of protein in the lipids can be attributed to two major causes. First, when double-extraction procedures (b) and (c) are used, there is a build-up of water in the extraction system which dissolves small quantities of protein; and secondly, when the material is reground, more degraded protein is formed, which is readily soluble in traces of water.

This second cause would explain the differences within group (a) of Table 1.

The average of the values obtained, where only traces of protein were present (eight determinations classified 'V. low' in Table 1), is $0.0673 \pm 0.0035\%$ (s.d.) of the dry ox-femur compact bone. The single value of 0.0629% obtained by the bulk extraction of compact bone from other ox-leg bones is in good agreement.

The cholesterol contents are also given in Table 1. The amounts of the cholesterol esters, calculated from the difference between total and free cholesterol, can be regarded as only approximate, as this difference is small.

The phospholipid content was 2.2% of the total lipid. The possible effect of contamination arising from the presence of inorganic phosphate is not known; however, the determination was carried out on re-extracted material. The total free fatty acids accounted for 71.5% of the Soxhlet-extracted material. The glycerol content of the cold-acetone-soluble lipid was 10.5%. From the average values obtained for free and total cholesterol contents the overall composition was calculated (Table 2).

The absorption spectrum of the lipid showed similarities to both serum β -lipoprotein (Oncley, Gurd & Melin, 1950) and pure β -carotene (Devine, Hunter & Williams, 1945). Thus a solution of the bone lipid in hexane exhibited well-defined peaks at 273 and 458 $m\mu$, a minor peak at 430 $m\mu$ and a shoulder at 485 $m\mu$, compared with pure β -carotene in cyclohexane which possesses peaks at 275, 456 and 482 $m\mu$ and a shoulder at 430 $m\mu$. Pure β -carotene in hexane, the complete absorption curve of which was not recorded, gave a major peak at 453 $m\mu$ (Devine *et al.* 1945).

Table 1. *Total lipid and cholesterol content of compact bone*

For methods of extraction (a), (b), (c) and (d), see Methods section.

Bone sample	Method of extraction	Total lipid (% of dry compact bone)	Protein content	Type of bone powder	Free cholesterol (% of total lipid)	Total cholesterol (% of total lipid)
Ox mixed bones	Cold acetone in bulk	0.0629	V. low	Once-ground material	10.9	12.3
Ox femur	(a)	0.0648, 0.0678	V. low	Once-ground material	13.3	14.3
		0.0638, 0.0697	V. low	Fraction from once-ground material passing 150-mesh	—	14.3
		0.0782, 0.0771	Low	Reground material passing 100-mesh	13.1	—
Ox femur	(b)	0.0987, 0.1012	High	Reground material passing 100-mesh	—	—
Ox femur	(c)	0.0718, 0.0802	Low	Reground material passing 100-mesh	—	—
Ox femur	(d)	0.0698, 0.0659 0.0737, 0.0627	V. low	Reground material passing 100-mesh	13.6	14.3

Table 2. *Composition of lipids from ox compact bone*

	Lipid extracted from ox-leg bones by cold acetone in bulk (g./100 g. of lipid)	Soxhlet-extracted lipid from ox femurs	
		(g./100 g. of lipid)	(g./100 g. of dry compact bone)
Triglycerides	85.5*	79.2	0.0535
Free cholesterol	10.9	13.3	0.0089
Cholesterol esters	2.4	1.7	0.0011
Phospholipids	Not determined	2.2	0.0015

* Not corrected for possible phospholipid content.

Serum β -lipoprotein in *cyclohexane* shows the same general shape of absorption spectrum as the bone lipid in hexane, possessing two well-defined peaks at 270 and 455 $m\mu$, a minor peak at 480 $m\mu$ and a shoulder at 420 $m\mu$.

DISCUSSION

The occurrence of protein-containing matter in some of the lipids isolated from bone has been discussed in the Results section. Its association with the lipid is partly due to the technique used for extraction. Its hydroxyproline content suggests that some 10% of the protein was derived from collagen (Eastoe, 1955); the remainder was probably derived from cellular material, either as free protein or in the form of an original lipoprotein.

The dry ox femur contained 0.0673% of lipid, and the other bones 0.0629%, by extraction with cold acetone in bulk. These lipids contained 13.3 and 10.9% of free cholesterol respectively, thus giving 0.0089 and 0.0069% of free cholesterol in compact bone. If it is assumed that bone contains 20.8% of organic matter (Eastoe & Eastoe, 1954), then the organic matter contains 0.042% of free cholesterol, a value considerably higher than the 0.0028–0.0040% found by Pikular (1955) in osseocollagen. The following possibilities are suggested to account for this difference: (a) most of the cholesterol–protein complex postulated by Pikular is dissociated during demineralization and the bulk of the cholesterol is lost; (b) there are two or more sources of cholesterol, the cholesterol–protein complex being a minor one; (c) the cholesterol is not in fact present as a protein–cholesterol complex in the matrix but as cellular material (possibly as protein–cholesterol complexes in the cells), the small quantity remaining in the tissue after demineralization being a residue of material, the bulk of which has been removed.

Some of the triglycerides present in the lipid might arise from the presence of chylomicrons or inclusions of bone marrow.

Lindemann (1957) studied the unusual dark-blue granules present in paraffin and celloidin sections of decalcified bone and teeth stained with haematoxy-

lin and eosin. She recalled that although these have been attributed either to calcium fluoride or to pathological calcification, she had been unable to detect calcium fluoride by an X-ray-diffraction technique. As a result of histochemical studies she suggested that the granules consisted largely of lipid material. It is possible that the lipids studied in the present work have their origin in the materials giving rise to the granules on staining. These lipids may have been derived from cellular material present in the bone.

Nikol'skaya (1953) has shown that myoalbumin, myoglobulin and myostromin from the muscles of a variety of mammals contain 0.039–0.120% of cholesterol, that found in myostromin being almost entirely in the free form. A similar state of affairs exists in compact bone, where 0.042% of the organic matter is free cholesterol and 0.005% is cholesterol esters.

SUMMARY

1. The lipid content of compact bone from ox femurs and from a mixture of other leg bones has been determined.
2. The isolated lipids have been examined and shown to contain triglycerides, cholesterol, cholesterol esters, phospholipids and probably β -carotene.
3. The free cholesterol content of compact bone has been shown to be higher than that of osseocollagen prepared from compact bone by demineralization. The reasons for this difference are discussed.
4. The relationship of the present investigation to earlier observations is examined.

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Uptake of Lead by Human Erythrocytes *in vitro*

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In the course of extensive studies on the effect of lead on erythrocytes *in vitro*, Aub, Fairhall, Minot & Reznikoff (1926) observed that whereas the cells became more fragile towards mechanical trauma they were more resistant to rupture by hypotonic solutions. Their experiments were made mainly on washed cells suspended in phosphate-free Ringer solutions, since the injurious effects were most manifest in such media.

It was postulated that precipitation of lead triphosphate took place on the erythrocyte membrane by reaction of lead salt with inorganic phosphate groups, with release of hydrochloric acid, and that points of localized acidity were responsible for increased fragility. Lead in whole blood was much less injurious than in phosphate-free Ringer suspensions, the difference being directly related to the inorganic phosphate content of the plasma. Any damage that was observed in cells in whole blood presumably arose by a simultaneous reaction of lead with the phosphate groups of the cell surface, which is largely dominated by such groups (Furchgott & Ponder, 1941). It follows that small quantities of lead added to whole blood should remain in the plasma as colloidal lead phosphate, whereas greater quantities would increase the fraction bound to the cells.

Certain aspects of this hypothesis have been discounted. Bischoff, Maxwell, Evans & Nuzum (1928) observed that lead salts of weak acids, e.g. lead glycerophosphate, produced changes in the erythrocyte similar to those with lead chloride, although no mineral acid was released. Bambach, Kehoe & Logan (1942) and Mortensen & Kellogg (1944) observed a marked uptake of lead by

erythrocytes even at low blood levels. The latter workers observed that small quantities of the radioisotope of lead were taken up rapidly by dog erythrocytes both *in vivo* and *in vitro*, from 90 % in 1 min. to a final level of 98 % at the end of 15 min. The process was reversible, and markedly dependent on temperature. At concentrations greater than 1 mg. of Pb^{2+} ions/100 g. of blood, the cells may be saturated and, at the maximum level, may contain only 35 % of the lead present.

The present work was undertaken to obtain more information on the interaction of lead and erythrocytes when brought into mutual contact. Our observations indicate that addition of lead salts to suspensions of erythrocytes in plasma, in isotonic sodium chloride or in Krebs-Ringer bicarbonate solutions results in the immediate formation of peptized lead phosphate sol. Combination with the cell occurs by coagulation or flocculation of the peptized sol on the cell surface.

EXPERIMENTAL

Materials

Blood from healthy young men was collected from the antecubital vein into a 0.9 % NaCl solution containing 1 g. of glucose/100 ml. and anticoagulant [2 mg. of heparin B.P. (Evans Medical Supplies Ltd., Liverpool) or 3 mg. of an ammonium oxalate-potassium oxalate mixture/10 ml. of blood]. The blood was used immediately after collection or stored at 4° and used within 48 hr.

The NaCl solutions used in this work were rendered lead-free by passage through a column of sulphonated polystyrene ion-exchange resin, Zeo-Karb 225 (The Permutit Co. Ltd., London, W. 4), in the sodium cycle. The solutions in the Krebs-Ringer bicarbonate mixture (Umbreit, Burris & Stauffer, 1951) were made lead-free in a similar manner, with the ion-exchange column prepared in the appropriate cycle.

The apparatus was of Pyrex glassware which was cleaned with boiling HNO_3 and thoroughly washed with doubly

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