Lipid Composition of Human Bone Marrow

By MARTHA WAJDA

Department of Biochemistry, Lister Institute of Preventive Medicine, London, S.W. 1

(Received 23 September 1964)

1. A modified method for the analysis of phospholipid mixtures by selective hydrolysis is described. 2. The phospholipid compositions of normal human bone marrow and of the bone marrows of patients who died with anaemia or various forms of leukaemia were investigated. 3. Phospholipids from normal bone marrow comprised about 44% of lecithin, 4% of choline plasmalogen, 7% of glyceryl ether phospholipid (choline base), 10% of sphingomyelin, 22% of phosphatidylethanolamine plus phosphatidylserine, 8% of ethanolamine plasmalogen and 5% of glyceryl ether phospholipid (ethanolamine base). 4. The proportion of kephalin (i.e. phosphatidylethanolamine plus phosphatidylserine) in the pathological bone marrows tended to be lower than normal. No other consistent differences were observed between the normal and pathological samples. 4. A ceramide dihexoside was isolated from normal bone marrow.

The lipid composition of bone marrow in various species of animal, including man, has been investigated by several workers (Pietruszko, 1962; Mulder, de Gier & van Deenen, 1962; Lund, Abadi & Mathies, 1962; Hallgren & Larsson, 1962), but little detailed information is available on the nature of the phospholipids, which form only a small proportion of the total lipid. Pietruszko (1962) found an unusually high proportion of a glyceryl ether derivative in the lecithin fraction, which was the main component of pig red bone marrow. Mulder *et al.* (1962) found that lecithin was the main component (50%) in red bone marrow of rabbit, cow and sheep; the proportion of glyceryl ether derivatives was not determined.

In view of the use of batyl alcohol as a therapeutic agent in leukaemia (Brohult & Holmberg, 1954) and in bracken poisoning (Evans, Evans, Thomas, Watkins & Chamberlain, 1958), the presence of glyceryl ether derivatives in bone marrow seemed of interest. In the present work the phospholipid composition of human bone marrow was investigated, particularly with regard to the presence of plasmalogen and glyceryl ether derivatives. The samples were obtained at autopsy, and were classified broadly as 'pathological', i.e. derived from patients with such diseases as leukaemia and anaemia in which some derangement might be expected, or 'normal', obtained from others in which there was no such suspicion.

MATERIALS AND METHODS

Red bone marrow, together with fragments of bone tissue, was removed from femurs and placed in chloroform-

methanol (1:1, v/v) within 1 hr. after autopsy. Before autopsy the bodies were kept at 4° for not more than 24 hr. after death.

Extraction of lipids. The bone-marrow samples, containing between 5 and 10 g. of tissue, which was dried and weighed after extraction of lipids, were extracted with 500 ml. of chloroform-methanol (1:1, v/v) followed by four further extractions with 500 ml. of chloroform-methanol (2:1, v/v). The combined extract was evaporated to dryness *in vacuo* at temperatures not exceeding 45°. The lipid residue was redissolved in 500 ml. of chloroform-methanol (2:1, v/v) and washed with 100 ml. of aq. 0.1 M-KCl. The aqueous phase was discarded, and the chloroform phase was washed three times with 100 ml. of aqueous-phase solvent chloroform-methanol-aq. 0.1 M-KCl (3:48:47, by vol.) and then evaporated to dryness; the lipid was redissolved in chloroform and dried over anhydrous Na₂SO₄. The lipid was dissolved in light petroleum (b.p. 40–60°), and neutral fats were separated from phospholipids and glycolipids by dialysis through a rubber membrane against the same solvent.

Analytical methods. Phosphorus, nitrogen, amino nitrogen and fatty aldehyde were determined by methods described by Gray & Macfarlane (1958). Hexose content was determined by the method of Dubois, Gilles, Hamilton, Roben & Smith (1956).

Paper chromatography. Water-soluble products of hydrolysis of phospholipids were chromatographed on Whatman no. 1 paper in butan-1-ol-acetic acid-water (5:4:1, by vol.) or phenol-acetic acid-ethanol-water (50:22:3:3, w/v/v/v). Phosphorus-containing compounds were located by the acid-molybdate spray of Hanes & Isherwood (1949) as modified by Dawson (1960). Sugar chromatograms were developed in butan-1-ol-pyridine-water (6:4:3, by vol.) and spots were located by spraying with AgNO₃-NaOH reagent.

Analysis of phospholipids by selective hydrolysis. The procedure of Dawson, Hemington & Davenport (1962) for

the identification of phospholipids by the products of mild alkaline and acid hydrolysis was modified to ensure the degradation of plasmalogens. The acyl group of these compounds is more resistant to mild alkaline hydrolysis than the corresponding group in diacylglycerophosphatides (Renkonen, 1963), and the intact plasmalogen on subsequent hydrolysis in 90% acetic acid gives free aldehyde and lysophosphatide without formation of cyclic derivatives. The reaction mixture was based on that of Hübcher, Hawthorne & Kemp (1960).

Step A: hydrolysis of monoacyl and diacyl-glycerophosphatides. The lipid sample (1.0–1.5 mg. of phosphorus) dissolved in 1.0 ml. of chloroform-methanol (2:1, v/v) was treated with 0.1 ml. of methanolic 0.5N-NaOH for 15 min. at room temperature. The reaction mixture was neutralized with ethyl formate, and mixed with 4.0 ml. of chloroform-methanol (2:1, v/v) and 1 ml. of water. The aqueous phase was removed, and the chloroform phase was washed three times with the aqueous-phase solvent chloroform-methanol-water (3:48:47, by vol.). The combined solution, hydrolysate A, containing the water-soluble products of hydrolysis of monoacyl- and diacyl-glycerophosphatides (GPE, GPS, GPC, GPI*), was analysed for phosphorus and amino nitrogen, and examined by paper chromatography.

Step B: hydrolysis of plasmalogens. The chloroform phase from step A was evaporated to dryness and treated with 1.0 ml. of aq. 90% (v/v) acetic acid for 1 hr. at 45°. Acetic acid was removed from the reaction mixture by azeotropic evaporation with carbon tetrachloride. The lipid residue, containing lysophosphatides derived from plasmalogens, was redissolved in 1.0 ml. of chloroform-methanol (2:1, v/v) and subjected to mild alkaline hydrolysis, as described in step A. The aqueous extract, hydrolysate B, containing the water-soluble hydrolysis products of plasmalogens (GPC, GPE), was analysed for phosphorus and amino nitrogen.

Step C: acid hydrolysis of sphingomyelin, glyceryl ether phospholipids and glycolipids. The chloroform phase from step B was evaporated to dryness, and the residue was hydrolysed with 1.0 ml. of 2N-HCl for 3 hr. at 100° in a ground-glass-stoppered tube. After cooling, 5.0 ml. of chloroform-methanol (2:1, v/v) was added, and after thorough mixing the resulting two phases were separated. The chloroform phase was washed three times with the aqueous-phase solvent chloroform-methanol-water (3:47:48, by vol.). The combined aqueous phase, hydrolysate C, was evaporated to dryness several times with small quantities of water until all the HCl was removed. The residue was redissolved in water and analysed for phosphorus and hexose.

The chloroform phase, containing glyceryl ether phosphate and sphingosine, together with fatty acids released during each step of the hydrolysis procedure, was analysed for phosphorus.

Calculations. The composition was determined from the analytical results expressed in $\mu\text{g. atoms}$, either directly or by difference on the basis of the following considerations:

(1) Original sample: P = total phospholipid; amino N = total ethanolamine and serine phospholipids; aldehyde = total plasmalogen.

* Abbreviations: GPE, glycerylphosphorylethanolamine; GPS, glycerylphosphorylserine; GPI, glycerylphosphoryl-inositol; GPC, glycerylphosphorylcholine.

(2) Hydrolysate A: P = total monoacyl- and diacyl-glycerophosphatides; amino N = ethanolamine and serine derivatives; P - amino N = choline and inositol derivatives.

(3) Hydrolysate B: P = total plasmalogen = original aldehyde; amino N = ethanolamine plasmalogen, P - amino N = choline plasmalogen.

(4) Hydrolysate C: (a) water-soluble fraction: P = phosphorylcholine = sphingomyelin; hexose = glycolipid. (b) Chloroform-soluble fraction: P = total glyceryl ether phospholipid.

Glyceryl ether phospholipid (ethanolamine base) = amino N of original sample minus the sum of the amino N in hydrolysates A and B.

Isolation of ceramide dihexoside. The lipid extracts of eight samples of normal bone marrows were combined. Neutral fats were removed by dialysis through a rubber membrane against light petroleum (b.p. 40–60°), and the non-diffusible lipids were hydrolysed with mild alkali and acetic acid, as described in steps A and B. The alkali- and acid-stable lipids, containing 6.6 mg. of lipid phosphorus and 13.3 mg. of hexose, were chromatographed on a column of Mallinckrodt silicic acid. Elution was started with chloroform, and followed by chloroform containing increasing concentrations of methanol. Eluate fractions were pooled after monitoring on thin-layer silica-gel-G (Merck) plates developed in chloroform-methanol-water (65:25:4, by vol.), and concentrated. Samples of concentrated fractions were hydrolysed in 2N-HCl for 3 hr. at 100°, and the water-soluble and chloroform-soluble products were analysed respectively for sugar and sphingosine and separated by paper chromatography.

Approx. 72% of the original sugar was eluted from the column with chloroform containing between 10 and 30% (v/v) of methanol. The concentrated fraction was still contaminated with phosphorus-containing compounds (approx. 1.0 mg. of phosphorus), which were quantitatively removed by passing the mixture through a Florisil column, and elution of glycolipids with chloroform-methanol (2:1, v/v). The purified glycolipid gave a hexose/sphingosine ratio 2:1. Paper chromatography of the hydrolysis products showed that glucose and galactose were the only sugar components, and were present in approximately equal quantities. Thin-layer chromatography of the intact glycolipid fraction on silica gel G in chloroform-methanol-water (65:25:4, by vol.) gave a double spot, with R_f values similar to those of an authentic sample of ceramide dihexoside kindly provided by Dr G. M. Gray, containing both the non-hydroxy and the hydroxy fatty acids (Svennerholm & Svennerholm, 1963).

RESULTS

The phospholipid content of normal human bone marrow (Table 1) appears to be related to the weight of fat-free dry residue, i.e. presumably to the cell content. However, the content of total lipid, which consists mainly of triglycerides, varied in the samples analysed from 0.6 to 4.4 g./g. of fat-free residue. The water content of the original samples was not determined but a similar variation was reported by Lund *et al.* (1962), who found the total lipid content of bone marrow to vary from 28 to 84% of tissue wet wt.

Table 1. *Lipid composition of 'normal' and pathological bone marrow*

Experimental details are given in the text.

Case no.	Age	Sex	Diagnosis	Total lipid (g./g. of fat-free residue)	Lipid phosphorus (mg./g. of fat-free residue)
1	73	Female	Chronic lymphatic leukaemia	0.36	0.71
2	79	Male	Chronic lymphatic leukaemia	4.64	0.79
3	74	Male	Acute myeloblastic leukaemia	0.84	1.09
4	46	Male	Hodgkin's disease	4.35	0.99
5	51	Female	Myelomatosis	—	—
6	54	Male	Myelomatosis (?)	4.33	0.23
7	74	Female	Myelomatosis and pernicious anaemia	4.45	0.95
8	71	Male	Macrocytic anaemia	1.12	1.75
9	71	Male	Aplastic anaemia	1.08	0.74
10	53	Female	Polymyositis	5.50	1.39
11	70	Male	Postgastrectomy malabsorption syndrome	0.21	0.34
Normal range				0.61-4.35	1.02-1.09

Table 2. *Phospholipid composition of 'normal' and 'pathological' bone marrow*

Experimental details are given in the text.

Case no. ...	Phospholipid composition (% of total)											Normal range
	1	2	3	4	5	6	7	8	9	10	11	
Monoacyl- and diacyl-glycero-phosphatides												
Choline base	52	59	43	62	55	47	47	43	57	57	54	38-52
Ethanolamine and serine bases	14	5	17	3	10	9	21	14	5	6	9	17-29
Plasmalogens												
Total	11	15	10	16	8	25	13	14	10	19	4	8-13
Choline base	—	11	5	—	3	—	6	0	7	13	1	2-5
Ethanolamine base	—	4	5	—	5	—	7	14	3	6	3	6-9
Glycerol ether phospholipids												
Total	11	14	19	6	14	6	10	15	17	14	14	9-16
Choline base	—	5	14	—	6	—	5	0	12	4	11	6-11
Ethanolamine base	—	9	5	—	8	—	5	15	5	10	3	2-8
Sphingomyelin	5	15	7	13	19	17	10	10	8	—	19	7-12
Total ethanolamine and serine derivatives	29	17	27	14	23	24	33	43	11	21	14	28-40

The phospholipid content of the 'pathological' bone-marrow samples was more variable, being lower than normal in six cases analysed, higher in two, and within the normal range in two.

Paper chromatography of the water-soluble products of hydrolysis of the phospholipids in general showed no differences between the compositions of the different samples.

Hydrolysate A contained GPC, GPI, GPE and GPS. The amounts of GPS were small and were estimated together with GPE by amino N determinations. Only traces of GPI were found, and were not separately estimated. No cardiolipin was observed.

Hydrolysate B generally contained GPC and GPE, arising from choline and ethanolamine

plasmalogens. In one pathological sample (case 8, macrocytic anaemia) no GPC was found. This agreed with the chemical analysis and indicated that no choline plasmalogen was present in that sample.

Hydrolysate C contained phosphorylcholine as the major water-soluble phosphoric ester. A small phosphorus-containing spot was located at the origin of the chromatograms developed in butan-1-ol-acetic acid-water (5:4:1, by vol.). This may originate from a minor unidentified phospholipid component of the tissue, but is possibly an artifact produced during the successive hydrolyses. Quantitatively the compound was ignored, and the amount of sphingomyelin was evaluated from the phosphorus content of hydrolysate C. Ethanol-

amine, arising from glyceryl ether phospholipid (ethanolamine base) was identified by spraying with ninhydrin. No serine was found.

In the hydrolysates from normal bone-marrow samples glucose and galactose were present in approximately equal quantities, together with traces of unidentified sugar components. The glycolipids of the pathological samples were not investigated. The quantitative analyses of the phospholipids are shown in Table 2.

DISCUSSION

Quantitative analyses (Table 2) of human bone-marrow phospholipids show that in normal tissue monoacyl- and diacyl-glycerophosphatides are the main components, accounting for 55–81% of the total phospholipid fraction, with about 40–50% of lecithin and 20–30% of kephalin (i.e. phosphatidyl-ethanolamine plus phosphatidylserine). Sphingomyelin (7–12%) and plasmalogens (8–13%) are also present. The main difference from other mammalian tissues is the presence of glyceryl ether phospholipids in comparatively large amounts (9–16%).

These results are in good agreement with those of Mulder *et al.* (1962), who investigated the phospholipid patterns of the bone marrows of rabbit, cow and sheep. However, detailed comparisons cannot be made, since the above workers reported only the contents of the main phospholipids in the tissue. The unidentified phospholipid, reported to be present in the bone marrow of pig epiphyses (Pietruszko, 1962), was not observed in human bone marrow.

Comparison of the phospholipid composition of normal and pathological bone marrow shows that in general the kephalin content of the pathological tissues was decreased. No other consistent difference was observed. The glyceryl ether phospholipid content varies, but in none of the samples

analysed falls markedly below or above the normal range.

Normal human bone marrow was found to contain glycolipids, the amount of lipid hexose being 0.5–2.2 mg./g. of lipid-free dry residue. The main glycolipid component (about 70% of the original sugar) was identified as a ceramide dihexoside, containing equal amounts of glucose and galactose (cf. cytolipin H; Rapport, Graf, Skipski & Alonzo, 1959).

I am indebted to the late Professor G. Payling-Wright for permission to use autopsy materials, and to study the post-mortem reports on the cases. The support of this work by the British Empire Cancer Campaign for Research is gratefully acknowledged.

REFERENCES

- Brohult, A. & Holmberg, J. (1954). *Nature, Lond.*, **174**, 1102.
Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
Dubois, M., Gilles, K. A., Hamilton, J. K., Roben, R. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Evans, W. C., Evans, A., Thomas, A. J., Watkins, J. E. & Chamberlain, A. G. (1958). *Brit. vet. J.* **114**, 180.
Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.
Hallgren, B. & Larsson, S. (1962). *J. Lipid Res.* **3**, 39.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hübscher, G., Hawthorne, J. N. & Kemp, P. (1960). *J. Lipid Res.* **1**, 433.
Lund, P. K., Abadi, D. M. & Mathies, J. C. (1962). *J. Lipid Res.* **3**, 95.
Mulder, E., de Gier, J. & van Deenen, L. L. M. (1962). *Biochim. biophys. Acta*, **59**, 502.
Pietruszko, R. (1962). *Biochim. biophys. Acta*, **64**, 562.
Rapport, M. M., Graf, L., Skipski, V. P. & Alonzo, N. F. (1959). *Cancer*, **12**, 438.
Renkonen, O. (1963). *Acta chem. scand.* **17**, 634.
Svennerholm, E. & Svennerholm, L. (1963). *Biochim. biophys. Acta*, **70**, 432.