[CONTRIBUTION FROM THE SEVERANCE CHEMICAL LABORATORY, OBERLIN COLLEGE]

The Isolation and Identification of Batyl Alcohol and Cholesterol from Yellow Bone Marrow[1]

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The victims of agranulocytosis or leucopenia lack the ability to increase automatically the normal number of white cells in the blood during the onset of infection. Since these phagocytes which combat invading infection are developed in the bone marrow it was fair to assume that administration of healthy marrow might be helpful. The use of yellow bone marrow in the treatment of this disease was described by Watkins and Giffen.[2a] Later, Marberg and Wiles[2b] showed that the therapeutic principle was concentrated in the unsaponifiable fraction of bone marrow.

It occurred to us that there was need for isolation of pure substances from the unsaponifiable fraction, with subsequent biological testing, in order to learn what substance might be particularly necessary to the production, maturing, and release of the phagocytes. Analysis, determination of structure, and synthesis might then give to medicine a useful weapon for attack on disease.

The yellow marrow from the larger bones of cattle was generously supplied by Swift and Company. The Abbott Laboratories rendered invaluable aid by preliminary saponification of this material on a substantial scale. The concentrate so prepared was re-saponified by us in order to make certain that no fats or other esters remained. The process was not unusual except, perhaps, in the use of isopropyl alcohol as solvent for the alkali (suggested by Dr. Charles Bills).

The fractionation of the non-saponifiable material was carried out according to the scheme given in Fig. 1. A methanol solution of the unsaponifiable matter was gradually cooled to dry-ice temperature and the colored crystals (P_1) isolated by low temperature filtration. The solid mass was then recrystallized from ethyl acetate (cooling to room temperature) and the precipitate (A) set aside for further purification. Filtrate (F_2) on cooling to 0° yielded a crop of crystals (P_3) which was recrystallized successively from methanol and from ethanol. The white crystalline material (B) thus obtained melted at 140–144°. Its further purification and examination will be described later in this paper.

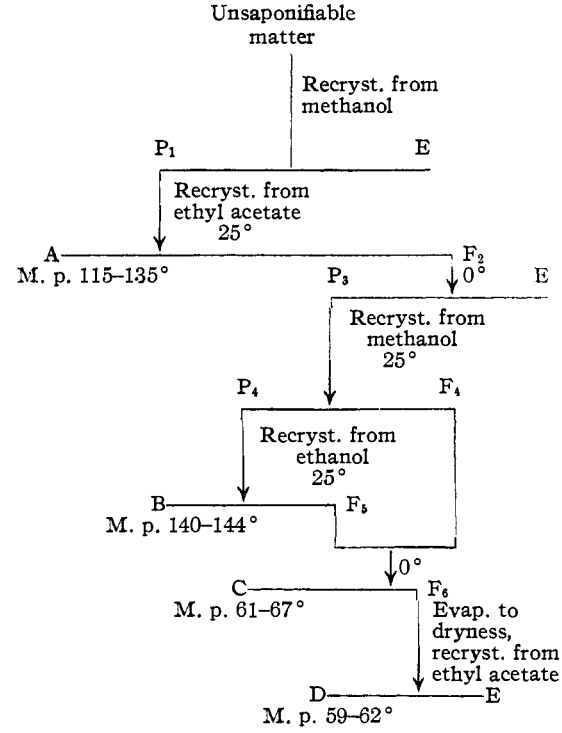

Fig. 1.—Fractionation of the Unsaponifiable Matter

The filtrate from fraction B was combined with F_4 and cooled to 0° whereupon yellow crystals (C) melting at 61–67° formed. Fraction C was subjected to solvent distribution between petroleum ether and methanol (containing 10% of water). The petroleum ether phase was evaporated to dryness and the residue further purified by chromatographic column separation using Norit carbon. The fraction most strongly adsorbed was recrystallized several times from aqueous ethanol and finally from aqueous acetone. The products separated as glistening white plates, m. p. 70–71°.

A fortunate chance comparison of this melting point with that of batyl alcohol, $HOCH_2$-$CHOH$-$CH_2O(CH_2)_{17}CH_3$, suggested possible identity. Toyama[3] reported a melting point of 70.4–71° for batyl alcohol; Davies, Heilbron and Owens[4] re-

(1) Presented before the division of Biological Chemistry of the American Chemical Society, St. Louis meeting, April, 1941.

(2) (a) Watkins and Giffen, *J. Am. Med. Assocn.*, **95**, 587 (1930); (b) Marberg and Wiles, *ibid.*, **109**, 1965 (1936).

(3) Toyama, *Chem. Umschau*, **31**, 63 (1924).

(4) Davies, Heilbron and Owens, *J. Chem. Soc.*, 2544 (1930).

ported 70–71°; while Nakamiya[5] found a melting point of 70.5–71°; all working with batyl alcohol isolated from shark liver oils. That the substance isolated in this Laboratory from yellow bone marrow was actually batyl alcohol was fully demonstrated by its physical and chemical properties and analysis, as well as by the characteristics of its *bis*-phenylurethan and *bis-p*-nitrobenzoate. While the melting point of 100–101° for the *bis*-phenylurethan agreed with that given for batyl-*bis* phenyl-urethan,[5,6] the *bis-p*-nitrobenzoate obtained in this Laboratory had a melting point of 65–66° rather than that of 53–54° reported by Heilbron and Owens[6] for batyl *bis-p*-nitrobenzoate containing two molecules of methanol of crystallization. Analyses showed that, in spite of repeated recrystallization from methanol, our product was free of solvent, hence the difference in melting points. In conformity with the structure assigned, treatment of batyl alcohol isolated from yellow marrow with hydriodic acid gave *n*-octadecyl iodide, identified by a mixed melting point with an authentic sample.

The mixed melting point of equal amounts of pure synthetic batyl alcohol (m. p. 70–71°) and the compound isolated from marrow was 67–69°. Davies, Heilbron and Owens[4] reported 68–69.5° as the melting point of a 1:1 mixture of their natural and synthetic batyl alcohol. That this depression in melting point was a consequence of mixing racemic batyl alcohol with one of its optical antipodes was shown by the finding that the crystalline alcohol isolated from marrow had a small, but definite, optical rotation, $[\alpha]^{20}D$ + 1.14°; Davies, Heilbron and Jones[7] reported $[\alpha]^{20}_{5461}$ + 2.6°. It appears probable, therefore, that the batyl alcohol isolated from yellow bone marrow is identical with that found in marine animals. The possibility that they are enantiomorphs has, however, not been rigidly excluded since the rotational values involved are very low and the differences in wave length and concentration might alter the sign of rotation. This is apparently the first time that batyl alcohol has been isolated from other than marine animals.

Fractions A and B were recrystallized repeatedly from ethanol until no further change in melting point was observed and then benzoylated. After a number of recrystallizations from ethanol and from ethyl acetate pure cholesteryl benzoate was secured, m. p. 145°, $[\alpha]^{23}D$ −13.4°. Shriner and Ko[8] reported a melting point of 144–145° and $[\alpha]^{25}D$ −13.7° for this substance. Saponification of the benzoate yielded analytically pure cholesterol, m. p. 147°, which, when acetylated, gave crystals melting at 113.5–114°. These constants are in good agreement with the accepted melting points of cholesterol and its acetate. The presence of cholesterol in yellow bone marrow has been taken for granted for some time. This assumption has, apparently, been based on the ubiquity of cholesterol in animal tissues and on positive sterol color reactions obtained with bone marrow. Beumer and Bürger[9] prepared cholesterol digitonide from a bone marrow concentrate. However, they made no definite statement of actual isolation of the pure free sterol.

Fractions D and E are now being investigated for the possible isolation and recognition of other pure substances.

Experimental Part

The yellow bone marrow[10] used in this investigation was obtained from the long bones of freshly slaughtered cattle, kept well refrigerated, and processed within two days. The moisture content of yellow bone marrow varies, approximating 10%. About 97% of the dry weight of the marrow consisted of fat, *i. e.*, ether and chloroform soluble material; the remaining 3% was a tan-colored, fibrous material which dissolved in isopropanolic potash on warming. The bone marrow fat contained about 1% non-saponifiable material. Phosphorus and carbohydrate could not be detected, so neither phospholipids nor galactolipids can be present in significant amounts.

Preliminary Saponification.[11]—Four and one-half kg. (10 lb.) of yellow bone marrow was refluxed for one hour with 14.5 liters (4 gal.) of isopropanol containing 1.4 kg. (3 lb.) of potassium hydroxide. The saponification mixture was cooled, diluted with 72 liters (20 gal.) of distilled water, and extracted with one 36-liter (10 gal.) and two 18-liter (5 gal.) portions of ether. The ether and isopropanol were removed from the extracts by evaporation on a steam-bath, and the residue dissolved in 3.6 liters (1 gal.) of ether. The ethereal solution was washed once with water and evaporated on a steam-bath until nearly all the solvent was removed. The residue consisted of a concentrate of non-saponifiable material, together with a little soap, unsaponified fat and some solvent.

Re-saponification of the Crude Concentrate.—The crude concentrates from five 4.5-kg. lots of yellow bone marrow were united, dissolved in 500 ml. of isopropanol containing 50 g. of potassium hydroxide and refluxed for one hour. The isopropanol was then removed from the saponification

(5) Nakamiya, *Bull. Inst. Phys. Chem. Research (Tokyo)*, **17**, 837 (1938); *C. A.*, **33**, 8175 (1939).

(6) Heilbron and Owens, *J. Chem. Soc.*, 942 (1928).

(7) Davies, Heilbron and Jones, *ibid.*, 166 (1933).

(8) Shriner and Ko, *J. Biol. Chem.*, **80**, 4 (1928).

(9) Beumer and Bürger, *Z. exptl. Path. Ther.*, **13**, 367 (1914).

(10) Supplied through the generosity of Swift and Company.

(11) We are indebted to The Abbott Laboratories for carrying out this operation.

mixture by distillation at 35–40° under diminished pressure. The residue was dissolved in 750 ml. of ether, and washed with water until neutral. The aqueous washings were twice reëxtracted, and these ethereal extracts were also washed until neutral. All the ethereal extracts were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness on a water-bath. The residue, which consisted of a viscous red-brown oil admixed with crystals, weighed 40 g.

Fractionation of the Unsaponifiable Material.—The fractionation procedure is outlined in Fig. 1. Forty grams of non-saponifiable material was dissolved in 400 ml. of boiling methanol, and the solution slowly cooled to dry-ice temperature. The colored crystals (P_1), so obtained, were isolated by filtration at dry-ice temperature. Upon dissolving P_1 in 150 ml. of boiling ethyl acetate and cooling to room temperature 3.8 g. of crystals (A) resulted, m. p. 115–135°. On cooling F_2, the mother liquor of A, to 0° another precipitate (P_3) was produced. This latter material (P_3) was fractionated from 250 ml. of methanol by stepwise cooling. The needles which separated at room temperature (P_4) were taken up in boiling ethanol and the resulting solution allowed to cool to room temperature. In this way 7.25 g. of material melting at 140–144°, fraction B, was secured. The methanol and ethanol filtrates F_4 and F_5 were combined and cooled to 0°. There was thus obtained 4.36 g. of crystals (C), m. p. 61–67°. A final crystalline fraction (D) was obtained by removal of the solvents from F_6 and recrystallization of the residue from ethyl acetate. Fraction D weighed 1.4 g. and melted at 59–62°. The viscous, red-brown oil which remained after removal of solvents from the various filtrates designated E weighed 20.0 g. This material is now being investigated.

Identification of Batyl Alcohol.—Fraction C was purified further by solvent distribution between petroleum ether (b. p. 35–60°) and methanol containing 10% of water. The process was repeated three times. The methanol-soluble material was consolidated with D while the petroleum ether phase was evaporated to dryness, the residue taken up in a 1–1 mixture of methanol–petroleum ether (b. p. 35–60°) and the solution passed through a column of Norit A (activated by heating at 350–400° in air for two hours). The first 20–25% of material to run through the column melted over the range 55–65°. By continued washing with the same solvent mixture the remainder of the solids was eluted; m. p. 65–70°. Upon re-columning the higher melting fraction, material melting at 67–70° was obtained. This was recrystallized twice from aqueous ethanol and then, for analysis, from aqueous acetone; glistening white plates, m. p. 70–71° (cor.); Davies, Heilbron and Owens[4] reported 70–71°; Toyama,[3] 70.4–71°; Nakamiya,[5] 70.5–71°.

Anal. Calcd. for $C_{21}H_{44}O_3$: C, 73.25; H, 12.79. Found: C, 73.25, 73.30; H, 12.50, 12.44. *Rotation.* 0.660 g. made up to 10 cc. with chloroform at 20° gave α_D +0.15°; l, 2; $[\alpha]^{20}_D$ +1.14°. Davies, Heilbron and Jones[7] reported $[\alpha]^{20}_{5461}$ +2.6°.

The melting point of a 1:1 mixture of this material and pure synthetic batyl alcohol (m. p. 70–71° cor.) was 67–69°. Davies, Heilbron and Owens[4] reported 68–69.5° for the mixed melting point of equal quantities of natural and synthetic batyl alcohol.

Refluxing 0.3 g. of batyl alcohol with 5 ml. of 57% hydriodic acid for two hours and working up the product as described by Heilbron and Owens[6] gave *n*-octadecyl iodide, m. p. 33–34° (cor.); the mixed m. p. with an authentic specimen of m. p. 33–34° (cor.) was 33–34° (cor.).

Batyl-*bis*-phenylurethan was prepared according to Heilbron and Owens,[6] m. p. 100–101° (cor.). These authors reported m. p. 98°; Nakamiya,[5] m. p. 101–101.5°.

Anal. Calcd. for $C_{35}H_{54}N_2O_5$: C, 72.16; H, 9.28; N, 4.81. Found: C, 72.01; H, 9.35; N, 4.88.

Batyl *bis*-*p*-nitrobenzoate was obtained on treating the alcohol with *p*-nitrobenzoyl chloride in the presence of pyridine. The dark red color noted by Heilbron and Owens[6] was not observed. Recrystallization from methanol gave pale yellow needles which soften at 63° and melt at 65–66° (cor.).

Anal. Calcd. for $C_{35}H_{50}N_2O_9$: C, 65.42; H, 7.79; N, 4.36. Found: C, 65.59; H, 7.97; N, 4.52.

Heilbron and Owens[6] reported a m. p. of 53–54° for batyl *bis*-*p*-nitrobenzoate containing two molecules of methanol of crystallization. In spite of repeated recrystallization from methanol our product was always solvent free.

Identification of Cholesterol.—Fractions A and B were recrystallized repeatedly from ethanol until the m. p. reached 143–146° at which point further recrystallization caused no change. The crude cholesterol was then converted to the benzoate and recrystallized two times from ethanol, twice from ethyl acetate and finally again from ethanol; m. p. 145°.

Rotation. 0.217 g. made up to 10 cc. with chloroform at 20° gave α_D −0.58°; l = 2; $[\alpha]^{23}_D$ −13.4°.

Shriner and Ko[8] reported a m. p. of 144–145° and $[\alpha]^{25}_D$ −13.7° for cholesteryl benzoate.

Upon saponification of the benzoate pure cholesterol was obtained; m. p. 147°. The m. p. values reported in the iterature vary from 146.5°[12] to 150–151°.[13]

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.9; H, 11.9. Found: C. 83.8; H, 12.2.

Finally, a portion of the purified cholesterol was converted to the acetate; m. p. 113.5–114°. The literature contains values ranging from 113°[14] to 115–116°.[13]

Fraction D.—This material was dissolved in a 1:1 mixture of methanol–petroleum ether (b. p. 35–60°) and passed through a column of activated Norit A. In this manner D was split into two main fractions, one composed of batyl alcohol and the other of a waxy product, m. p. 55–60°. This material is now being investigated.

Summary

1. Batyl alcohol, $HOCH_2$-$CHOH$-CH_2-O-$(CH_2)_{17}$-CH_3, hitherto found only in marine organisms, has been isolated from the yellow bone marrow of cattle.

2. Cholesterol also has been isolated from yellow bone marrow.

3. This work is being continued in an effort to obtain other pure substances from marrow.

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(12) Diels and Linn, *Ber.*, **41**, 260 (1908).
(13) Anderson, *J. Biol. Chem.*, **71**, 407 (1927).
(14) Rayman, *Bull. soc. chim.*, [2] **47**, 898 (1886).