CCVII. CALOSTEROL, A STEROL PRESENT IN THE MILKY JUICE OF *CALOTROPIS GIGANTEA*.

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IN the course of our investigations on the proteolytic enzymes in the milky juice of *Calotropis gigantea* [Basu and Nath, 1933] our attention was drawn to the large amount of ether-soluble substances in the dried juice which gave the Liebermann-Burchard test with acetic anhydride and sulphuric acid. By working up the unsaponifiable matter of the ether-soluble fraction it has been possible to isolate a new sterol which it is proposed to call calosterol. The sterol melts at 202–203° and has therefore a higher melting-point than that of any known sterol [*cf.* Wieland and Gough, 1930] with the exception of cerevisterol [Bills and Honeywell, 1928; Honeywell and Bills, 1932] which melts at 265·3°. Calosterol has the formula $C_{28}H_{44}O$ and thus appears to be isomeric with ergosterol but is dextrorotatory and possesses the high specific rotation in chloroform of $[\alpha]_{5461}^{24} = +100\cdot6$. It appears to contain three double bonds. It is not precipitated by digitonin.

Preparation and purification.

85 g. of residue obtained by drying about 425 ml. of the milky juice at the ordinary temperature *in vacuo* were extracted with sodium-dried ether in a Soxhlet apparatus and 33·3 g. of ether-soluble matter obtained. The latter was then saponified with 3 % alcoholic KOH and freed from the insoluble soap by filtration. The alcohol was removed by evaporation on a water-bath and the residue extracted with ether. About 20 g. of unsaponifiable matter were thus obtained. It was crystallised four times from absolute alcohol and from alcohol-benzene mixture (2 : 1). We tried the method of benzoylation and subsequent saponification as adopted by Wieland and Asano [1929] but without success. We therefore repeatedly (six times) crystallised from acetone and each time determined the melting-point and the specific rotation. Ultimately 5·96 g. of seemingly pure crystals of a sterol of M.P. 202–203° and specific rotation $[\alpha]_{5461}^{24} = +100\cdot2°$ were obtained. To ensure the greatest possible purity of the sterol it was then benzoylated according to the method recommended by Callow [1931], the sterol being recovered by saponification.

Calosterol benzoate. 3·5 g. of the substance were dissolved in 20–25 ml. of redistilled pyridine, cooled in ice and treated with benzoyl chloride drop by drop until a permanent pink colour was obtained. The mixture was allowed to stand for 5 hours when crystals of the benzoate (fraction I) were obtained. These were collected, washed with a little pyridine and the filtrate cooled to −4°, when a second fraction of crystals was obtained. Fractions I and II were separately washed with water and then with alcohol. The aqueous washings from the two

crops were added to the filtrate and the precipitate (fraction III) thus obtained was collected and washed.

Fraction I	2·03 g.	M.P. 237–239°	$[\alpha]_{5461}^{24} = +121\cdot0°$
Fraction II	0·85 g.	M.P. 208–225°	$[\alpha]_{5461}^{24} = +118\cdot6°$
Fraction III	0·61 g.	M.P. 199–206°	$[\alpha]_{5461}^{24} = +114\cdot0°$

These fractions were then worked up as recommended by Callow until no further alteration in specific rotation took place. The calosterol benzoate thus obtained had M.P. 239–240° and specific rotation $[\alpha]_{5461}^{24} = +121\cdot6°$.

(Found: C, 83·74, 83·80; H, 9·67, 9·51 %. $C_{35}H_{48}O_2$ requires C, 84·00; H, 9·60 %.)

Calosterol. 1·5 g. of the pure benzoate were saponified with about 70 ml. of 3 % alcoholic KOH for about half an hour. The sterol separated on pouring the mixture into water and was filtered by suction, washed with water and dried in a vacuum. It was then crystallised successively from acetone, methyl alcohol and absolute alcohol. The pure sterol had M.P. 202–203° and a specific rotation of $[\alpha]_{5461}^{24} = +100\cdot6°$ in chloroform.

(Found: C, 84·88, 84·74; H, 10·54, 10·64 %. $C_{28}H_{44}O$ requires C, 84·84; H, 11·11 %.)

Calosterol crystallises well from acetone, alcohol and from alcohol-benzene mixture (2 : 1). The sterol is very soluble in ether but only dissolves in about 200 parts of boiling alcohol.

Calosterol is not precipitated by digitonin and thus resembles lanosterol and agnosterol [Lifschütz, 1926] and also cerevisterol [Honeywell and Bills, 1933].

Calosterol is quite stable. Exposed to the air and light of the laboratory for several weeks, it showed no discoloration or change in melting-point.

Calosterol acetate. 0·3 g. of the purest calosterol and 5 ml. acetic anhydride were boiled under reflux for 15 mins., cooled, poured into water and filtered. The acetate was washed, dried and crystallised twice from glacial acetic acid; yield 0·2 g. M.P. 211–212° and specific rotation $[\alpha]_{5461}^{24} = +105\cdot0°$ in chloroform.

(Found: C, 82·21, 82·12; H, 10·34, 10·15 %. $C_{30}H_{46}O_2$ requires C, 82·19; H, 10·50 %.)

From the analyses of the acetate and the benzoate it is quite clear that calosterol contains one hydroxyl group. It will be seen that the acetate and the benzoate show higher specific rotations than the calosterol itself. While it is unusual for a sterol ester to exhibit greater optical activity than the parent sterol, a similar behaviour has been noted with cerevisterol [Honeywell and Bills, 1932] and *iso*ergosterol [McDonald and Bills, 1930].

Calosterol hydrate. The sterol crystallises with one molecule of water of crystallisation from 90 % alcohol and retains this water even *in vacuo*.

(Found: C, 81·03; H, 11·18 %. $C_{28}H_{44}O, H_2O$ requires C, 81·16; H, 11·11 %.)

The water could, however, be removed by heating the hydrate to 110° for 2 hours and then drying it *in vacuo*.

With regard to the microanalytical data given above it should be noted that a large number of analyses were made and concordant results obtained. Only a few typical data are given. That the apparatus was functioning correctly was ensured by analysing from time to time a pure sample of cholesterol (Pfanstiehl) when correct values for carbon and hydrogen were obtained.

Colour reactions of calosterol.

With the Liebermann-Burchard reagent calosterol gives a reddish violet colour which changes to violet, then quickly to blue and finally to green.

Tortelli-Jaffe's reaction gives a bright yellow colour.

In Salkowski's reaction the chloroform layer is colourless and the sulphuric acid layer is coloured orange-yellow and gives a green fluorescence.

With trichloroacetic acid as well as with chloral hydrate [Rosenheim, 1929] calosterol remained colourless.

With regard to its different colour reactions calosterol thus differs from ergosterol and any other known sterol.

Unsaturation.

Using the method of Reindel and Niederlander [1929] experiments were carried out with cholesterol as well as with calosterol. Table I shows the results.

Table I.

1 Substance	2 Wt. of substance g.	3 $N/10$ Br in CCl$_4$ added ml.	4 $N/10$ Br in excess ml.	5 $N/10$ Br transformed to HBr in blank ml.	6 $N/10$ Br required to saturate double bond ml.	7 $N/10$ Br required for each double bond ml.	8 No. of double bonds found
Cholesterol	0·1081	16·35	·7·55	1·55	7·25	5·22	1·4
Calosterol	0·0757	34·18	17·98	3·37	12·83	3·93	3·3

The number of double bonds in calosterol thus appears to be three. The slightly higher values obtained might be caused by the bromine solution partially oxidising the OH group and forming hydrobromic acid. Estimation of hydrobromic acid (by adding a solution of potassium iodate and titrating the liberated iodine with $N/10$ sodium thiosulphate) however showed that while the HBr formation in the case of cholesterol was such that by taking that amount into consideration the number of double bonds obtained fell from 1·4 to 1·0, a very large amount of HBr was found in the case of calosterol. This was probably caused by the liberation of HBr by the brominated sterol in presence of water.

Catalytic hydrogenation. As catalyst platinum black prepared according to the method of Willstätter and Waldschmidt-Leitz [1921] was employed. The solvent used was glacial acetic acid containing a trace of hydrochloric acid at temperatures of 60–70°. Calosterol was employed in the form of its acetate, parallel experiments being carried out with cholesterol acetate. The amount of catalyst employed was 0·1 g. Table II shows the results.

Table II.

Substance	Weight in g.	Period of shaking hrs.	Vol. of H$_2$ absorbed at N.T.P. ml.	Vol. of H$_2$ at N.T.P. required to saturate one double bond ml.	No. of double bonds found
Cholesterol acetate	0·2012	4	10·51	10·52	1·0
Calosterol acetate	0·0975	6·5	10·93	4·98	2·2

Thus by catalytic hydrogenation the number of double bonds found is two, which is one less than the number obtained by the method of bromination. Similar behaviour is shown by ergosterol as well as by zymosterol.

Calosterol, $C_{28}H_{44}O$ with three double bonds, thus appears to be isomeric with ergosterol, which has recently been shown by Windaus and Luttringhaus [1932] to have the formula $C_{28}H_{44}O$.

SUMMARY.

Calosterol, a stable sterol isolated from the milky juice of *Calotropis gigantea*, has the formula $C_{28}H_{44}O$. It melts at 202–203° and has a specific rotation in chloroform of $[\alpha]_{5461}^{24} = +100 \cdot 6°$. The sterol is not precipitated by digitonin. The benzoate melts at 239–240° and has $[\alpha]_{5461}^{24} = +121 \cdot 6°$. The acetate melts at 211–212° and has $[\alpha]_{5461}^{24} = +105 \cdot 0°$. The method of bromination gives three double bonds for calosterol while catalytic hydrogenation with platinum black yields only two.

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