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2. *Ibid.*
3. Guelpa, *Bulletin General De Therapeutique*, 1909, Vol. 157, pp. 91 and 770; *Ibid.*, 1910, Vol. 159, p. 213.
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ON CHOLESTERYL PALMITATE, A CONSTITUENT OF CORPUS LUTEUM.*

BY MERRILL C. HART AND FREDERICK W. HEYL.

Cholesterol and cholesteryl esters are extremely important physiological substances and one or both are found in nearly all living tissue. The cholesteryl esters are a very labile cell constituent and vary greatly under different pathological conditions. Thus in some cases the cholesteryl esters of the human suprarenal capsule are increased 5 to 7 fold and under others they are decreased to one-tenth of their normal amount, the content of free cholesterol remaining approximately the same. An increase in the ester content is also observed in chronic kidney disease, diabetes and pregnancy and a decrease in infection.[1] The cholesteryl ester content of the suprarenal capsule is also influenced by the injection of saponin into the blood stream.

Laudau and McNee[2] showed that in man the entire cholesterol content of the adrenals is decreased in phthisis, other infections and neoplasms, while it is increased in inanition, pedatrophy and circulatory disturbances. The variations are chiefly of the esters. These experiments seem to indicate that the adrenals are a storehouse for cholesteryl esters but are not producers of cholesterol. They are regarded as intermediatory organs for cholesterol metabolism. The liver, bone marrow, lymph glands and spleen also appear to have this function.

Kondo[3] showed that 39 per cent. of the total cholesterol of the liver was in the ester form. Lapworth[4] obtained cholesterol and cholesteryl esters from kidneys, the adrenals, a dermatoid cyst and the brain. More than 99 per cent. of the brain cholesterol was found to be uncombined.

That the cholesteryl ester of the blood varies greatly was shown by the work of E. Hermann and J. Newmann[5] who found in 1 kilogram of normal female blood

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[1] Wacker and Hueck, *Archiv. f. exper. Pathol. u. Pharmacol.*, 71, 373, 1913.

[2] *Beitr. path. Anat.*, 58, 667, 1914.

[3] *Biochem. Zeit.*, 26, 238, 1910.

[4] *J. Path. Bact.*, 15, 254, 1911.

[5] *Biochem. Zeit.*, 43, 47 1912.

0.8641 Gm. of free cholesterol and 0.5755 Gm. of cholesterol as ester. In pregnant women the figure was 0.8346 for the free cholesterol and 0.9708 for the cholesteryl ester. In the blood of the new born, while the content of free cholesterol was 0.7811 Gm., the cholesterol as ester was only 0.1413 Gm.

Cholesteryl esters have been found in many organs. The normal kidney contains 0.012 to 0.03 per cent. of which 25 per cent. is cholesteryl palmitate and 75 per cent. cholesteryl oleate.[1] The normal aorta[2] contains 0.032 to 0.047 per cent. and L. Wacker and W. Hueck[3] found 0.10 to 0.15 Gm. in the normal suprarenal capsule. Wet blood corpuscles of the ox[4] contained 0.044 per cent. free cholesterol and 0.0019 per cent. of cholesteryl ester while sheep corpuscles gave 0.0589 per cent. free cholesterol and 0.0689 per cent. cholesterol in ester form.

Cholesterol as an ester was also found by K. Kauders[5] in 1 liter of the blood serum of the following animals in the amounts given: horse, 0.0199 Gm.; dog, 0.03285 Gm.; ox, none; rabbit, 0.2946 Gm.; guinea pig, 0.2364 Gm.; sheep, 0.038 Gm.

Cholesteryl esters have also been found in the dry placenta[6] varying from 0.072 Gm. in the normal placenta to 0.751 Gm. in the premature placenta. Bloor and Knudson[7] have shown that in the whole blood of normal individuals, the cholesterol combined as ester constitutes about 34 per cent. of the total cholesterol and that in the plasma was about 58 per cent. A variation in different individuals of about 15 per cent. of the average with no marked difference between men and women was found. In pregnancy, the percentage of cholesterol combined as ester was high and in nephritis and carcinoma it was low.

Cholesteryl palmitate has been found in the blood serum,[8] in the suprarenal capsule,[9] in the skin[10] and in the doubly refracting material from pathological organs.

The melting points of these cholesteryl esters have not the significance as to purity usually assigned to them because of the properties of these esters of forming liquid crystals and existing in several phases.[11]

Previous analytical work on corpus luteum on an ether extract had shown the presence of 0.70 per cent. free cholesterol and of 0.92 per cent. of cholesterol in the form of the ester. By extraction by means of acetone in the study of the lipoid constituents of corpus luteum, which will be reported on later, it was possible to isolate cholesterol in the form of its palmitic acid ester. As a quantitative yield of the esters was not obtained, there is the possibility of the existence of other cholesteryl esters in this gland.

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[1] A. Windhaus, *Zeit. f. physiol. Chem.*, 65, 114, 1910.

[2] A. Windhaus, *Ibid.*, 67, 174, 1910.

[3] *Archiv. f. exper. Pathol. u. Pharmacol.*, 71, 373, 1913.

[4] K. Kauders, *Biochem. Zeit.*, 55, 96, 1913.

[5] *Loc. it.*

[6] B. Bienenfeld, *Biochem. Zeit.*, 43, 245, 1912.

[7] *J. Biol. Chem.*, 29, 7–13, 1917.

[8] Hurthle, *Zeitschr. f. physiol. Chem.*, 21, 342, 1895–1896.

[9] Rosenheim and Tebb, *Jour. of Physiol.*, 38, Proc. 2, 1909.

[10] Salkowski, *Arbeiten. ad. Pathol. Inst. zu. Berlin*, 2, 1906.

[11] Jaeger, *Recueil des travaux chim. des Pays-Bas*, 25, 334, 1906; Waterant, *Compt. rend.*, 143, 605, 1906; Lehmann, *Zeit. f. angew. Chem.*, 1641, 1906; A. Prins, *Zeit. f. phys. Chem.*, 67, 639, 1909; P. Gaubert, *Compt. rend.*, 149, 608, 1909; C. White, *Proc. Phys. Soc.*, VI, 38, 1908.

EXPERIMENTAL.[1]

The sample of corpus luteum used in the following experiments represented carefully collected, hand-dissected material, dried immediately after collection in a high vacuum and below 40° C. in the commercial way. The dried corpus luteum represents approximately 6 parts of the fresh corpus luteum substance. It was representative of the material found on the pharmaceutical market in that it had been taken from such a large number of cattle as to represent a good average sample. It contained 4.5 per cent. moisture and 5.4 per cent. ash.

The material was ground to pass through a No. 20 sieve. One kilogram of this powder was thoroughly extracted thrice with 6 liters of pure acetone.

The first acetone extract was concentrated under reduced pressure in an atmosphere of nitrogen to a volume of approximately 1 liter. On standing for several weeks at 0° C. a white solid separated, which was filtered off, washed with a little acetone and dried *in vacuo*. This weighed 2.44 Gm. This melted very indefinitely, softening and becoming vaseline-like at 68 to 71° C. and not forming a clear oil till about 130° C. Nothing more of a crystalline nature was obtained from the filtrate from this on further concentrations.

The second acetone extract yielded when treated in a similar manner 0.91 Gm. more of a substance with like properties. The third acetone extract gave 0.64 Gm. of this substance.

This crystalline material was fractionally crystallized from hot absolute alcohol. The top insoluble material after repeated crystallizations gave very uniform microscopic flat, plate-like crystals melting at 71 to 73° C. to a vaseline-like consistency. The microscopic appearance or the melting point was not influenced by further crystallization from alcohol. This material (Fraction A) was insoluble in water, very sparingly soluble in cold alcohol and extremely soluble in ether, benzene, chloroform and hot alcohol. It gave the Liebermann-Burchard test for cholesterol. It was phosphorus-free, showing the absence of lipoid material. After drying *in vacuo* this material weighed 1.15 Gm. This substance was optically active.

Subs., 0.1562 in 10 cc. redistilled chloroform gave a rotation of $-1.1°$ V. in a 1-dcm. tube. Hence $[\alpha]_D^{20} = -24.4$.

These results indicate the possibility of this material being of the nature of a cholesteryl ester. It was saponified by boiling with alcoholic potash for 3 hours under a reflux condenser.

Subs., 0.7414 required on saponification, $N/20$ KOH, 24.27 cc. Calc. for $C_{27}H_{45}O\text{-}CO.C_{15}H_{31}$: $N/20$ KOH, 23.73 cc.

The alcohol was distilled off from the saponified material after being made distinctly alkaline with sodium hydroxide. The aqueous residue was thrice extracted with ether. A troublesome emulsion formed which was broken by means of the centrifuge. The ether solution was washed, dried and evaporated. 0.4288 Gm. of crystalline material melting at 143–145° C. was obtained. 0.0167 Gm. more of

[1] We are indebted to Dr. George E. Miller for some of the preliminary work on the isolation and identification of the following crystalline material.

cholesterol was obtained in the purification of the fatty acids, making a total of 0.4395 Gm. (Calculated yield of cholesterol from 0.7414 Gm. of cholesteryl palmitate is 0.4586 Gm.) Crystallization from alcohol gave 0.3326 Gm. of the typical cholesterol crystals melting at 146° C. This material gave the Liebermann-Burchard test for cholesterol. A mixed melting point with pure cholesterol (Pfahnstiehl) was not depressed. It was optically active.

Subs., 0.2096 in 10 cc. of redistilled chloroform gave a rotation of −2.13° V. in a 1-dcm. tube. Hence $[\alpha]_D^{20} = -35.2$.

The alkaline solution after the removal of the cholesterol by ether extraction was cooled in ice water and precipitated with dilute nitric acid. A white flocculent fatty acid precipitate formed which was filtered off, washed with cold water and dried. The filtrate from this showed the absence of any of the more soluble fatty acids.

The fatty acids were taken up in ether and extracted from this with 5% sodium carbonate solution. From the ether solution there was obtained 0.0167 Gm. more of cholesterol. The sodium carbonate extracts were precipitated in the cold with dilute nitric acid, the precipitated fatty acid filtered off and washed with cold water. Dried to constant weight *in vacuo* this acid weighed 0.300 Gm. (Calculated yield of palmitic acid from 0.7414 Gm. of cholesteryl palmitate is 0.304 Gm.)

This acid was crystallized from 7 cc. of alcohol. The separated fatty acid was cooled, filtered, and washed with a little cold alcohol. Dried to constant weight *in vacuo* this fraction weighed 0.1273 Gm. This melted at 60–61° C. and a mixed melting point with pure palmitic acid was not depressed. This was analyzed.

Analysis. Subs., 0.1223; H_2O, 0.1391; CO_2, 0.3366. Calc., for $C_{16}H_{32}O_2$: C, 75.0; H, 12.5. Found: C, 75.0; H, 12.7.

The filtrates from this fraction on concentration and treatment with a little water yielded 0.1583 Gm. more of this acid melting at 59 to 61° C.

Therefore, Fraction A consisted of pure cholesteryl palmitate.

There appeared to be in the filtrates from the above-described cholesteryl palmitate a slightly more soluble derivative with a higher melting point. The solvent was removed from these filtrates and the residue, after a thorough extraction with benzene to remove cholesteryl palmitate, was crystallized from alcohol.

As there was an evident lack of material for carrying on this work 600 Gm. more of the corpus luteum were extracted in a similar manner. The crystalline compound (3.40 Gm.) separating in this case from the acetone extract yielded 0.640 Gm. of a material insoluble in hot alcohol and decomposing at 268° C. The nature of this material will be reported on later. The cholesteryl palmitate was separated from this fraction soluble in hot alcohol by crystallization from alcohol and the filtrates added to the above fraction.

The residue from the benzene extract of the above residue was fractionated from alcohol. A slight amount more of cholesteryl palmitate was obtained. No indication of the presence of free cholesterol was to be found in the filtrates from this.

The residue insoluble in benzene weighed 0.69 Gm. and melted at 100 to 135° C. (material sintered at 100°, became vaseline-like at 104° and did not run down to a clear oil till about 135° C.). This material was separated into an ether-soluble and an ether-insoluble fraction by thorough treatment with 40 cc. of ether. The residue from the ether extract on crystallization from alcohol gave a slight amount of crystalline material melting at 80 to 81° C. This was of importance as indicating the possible presence of cholesteryl stearate which melts at 82° C.[1]

There was not sufficient material for further work on this.

The ether-insoluble fraction was then crystallized thrice from 20 cc. of hot alcohol. The last two crystallizations did not affect the melting point (104–135° C.) and the microscopic appearance of the crystals (flat plates). Dried *in vacuo* this fraction weighed 0.5215 Gm.

This material was taken up in 75 cc. of hot water, filtered, cooled and precipitated with dilute hydrochloric acid. A white flocculent fatty acid precipitate was obtained. This was filtered off, washed with cold water and dried *in vacuo*. It weighed 0.4602 Gm. This was fractionated from alcohol into three fractions weighing 0.3210, 0.1243, and 0.0149 Gm., respectively. The top fraction melted at 69–69.5° C. and a mixed melting point with pure stearic acid was not depressed. This was analyzed:

Analysis. Subs., 0.1599; H_2O, 0.1836; CO_2, 0.4448. Calc. for $C_{18}H_{36}O_2$: C, 76.0; H, 12.7. Found: C, 75.9; H, 12.8.

The fraction under this melted at 67–68° C.

The aqueous filtrate and washings from the above separation of stearic acid were evaporated to dryness in a platinum evaporating dish and ignited at a dull red heat. 0.0616 Gm. of material was obtained. This was analyzed for potassium by the Lindo-Gladding method[2] and gave 0.1843 Gm. of K_2PtCl_6 equivalent to 0.0566 Gm. of potassium chloride. This corresponds to approximately one-half the potassium that should be obtained from 0.5215 Gm. of normal potassium stearate.

These figures suggest that this material is of the nature of potassium bistearate, $C_{18}H_{35}O_2K + C_{18}H_{36}O$.[3]

The filtrates from the above-mentioned potassium stearate were gathered together, the solvent removed and the residue (0.9679 Gm.) saponified. This fraction showed on titration the presence of 0.4067 Gm. of free acid calculated as stearic. On hydrolysis it gave 0.1168 Gm. of alcohol and ether-soluble unsaponifiable matter giving the Liebermann-Burchard test for cholesterol. The fatty acid fraction gave on crystallization from alcohol 0.3056 Gm. of a mixture of palmitic and stearic acids melting at 60–61° C. A mixed melting point with pure palmitic acid was 57–58° C. Volatile fatty acids were absent.

SUMMARY.

The crystalline material separating on the concentration of the acetone ex-

[1] "Lewkowitsch Chem. Tech. and Anal. of Oils, Fats and Waxes," p. 66.

[2] *Bull. 107, U. S. Dept. of Agr.*, p. 11.

[3] "Lewkowitsch. Chem. Tech. and Anal. of Oils, Fats and Waxes," p. 128.

tracts of corpus luteum consists of cholesteryl palmitate, potassium stearate, possibly a very slight amount of cholesteryl stearate and free fatty acids.

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THE ANTISPASMODIC ACTION OF CHLORETONE.*

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Several years have now passed since Macht and his co-workers began their systematic pharmacological and clinical study of the action of the benzyl compounds—particularly benzyl benzoate and benzyl alcohol. In a series of articles beginning with his study of one group of the opium alkaloids Macht[1] logically deduced from his study of the other group that the benzyl component of the molecule must be responsible for the inhibitory and tonus lowering properties of the group as represented by papaverin. His subsequent investigation[2] of benzyl benzoate, benzyl acetate, benzyl alcohol[3] and finally benzyl mandelate[4] seemed to prove that in these compounds he had discovered very active antispasmodics whose therapeutic value was greater than any such drug previously discovered.

In considering the work of Macht on the benzyl compounds it seemed that other active sedatives had been largely overlooked. Knowing the powerful action of chloretone (tri-chlor-tertiary butyl alcohol, $C_4H_7OCl_3$) as a sedative, hypnotic antiseptic, local anesthetic and general anesthetic, it seemed more than probable that it would possess marked antispasmodic action as well. Accordingly a long series of experiments were begun in which a direct comparison could be made between the action of benzyl alcohol and chloretone. The sister bromine compound to chloretone, namely, brometone, was also included in part of the work. Benzyl alcohol was chosen for comparison because of the insolubility of the esters and because it is generally agreed that the esters are hydrolyzed into benzyl alcohol during the process of absorption from the alimentary tract and that it is consequently the action of benzyl alcohol which is observed.

ACTION ON ISOLATED SMOOTH MUSCLE.

The first series of experiments were conducted upon strips of isolated smooth muscle such as the uterus, intestine and ureter, but particularly the uterus of the virgin guinea pig.

A summary of six successful experiments on the isolated uterus of the guinea pig gave the following comparisons as to amounts of the substances necessary to inhibit the normal tonus of the muscle.

On 9/12/22, 2 cc. of 0.5% chloretone solution added to 100 cc. of Locke's solution was much more effective than 2 cc. of 1% benzyl alcohol solution and about as effective as 5 cc. of 1% benzyl alcohol solution. In this experiment chloretone was 5 times as effective as benzyl alcohol.

On 9/15/22, 1 cc. of 0.5% chloretone solution was equivalent to 1 cc. of 2% benzyl alcohol solution and chloretone was therefore 4 times as effective as the benzyl alcohol. (See Fig. 1.)

On 9/19/22, 2 cc. of 0.5% chloretone solution added to 100 cc. of Locke's solution was more effective than 2 cc. of 2% benzyl alcohol solution but 1 cc. of 0.5% chloretone solution was less

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* Scientific Section, A. Ph. A., Asheville meeting, 1923.