XI. LIGNOCERIC ACID FROM "CARNAUBON."

BY OTTO ROSENHEIM AND HUGH MACLEAN.

*From the Physiological Laboratory, King's College, London, and the
Pathological Chemical Laboratory, St Thomas's Hospital, London.*

(Received February 8th, 1915.)

Nearly ten years ago Dunham [1905] discovered that ox kidneys contained a substance resembling the so-called "protagon" which Liebreich had found in brain. On comparing the two substances prepared by identical methods from brain and from kidneys (extraction of the fresh tissue with 85 % alcohol at 45°, or with boiling alcohol after the coagulation of the proteins by 5 % sodium sulphate solution), he found that "the substance from the kidney contains distinctly more nitrogen and phosphorus than that from the brain," but that "the cleavage products show that all of these substances belong to the same group."

Later on, however, Dunham [1908] succeeded in isolating from the kidney-"protagon"¹ an acid of the composition $C_{24}H_{48}O_2$ which he considered identical with carnaubic acid. As this fatty acid of vegetable origin had not been previously found in animal tissues, Dunham and Jacobson [1910] gave the name "carnaubon" to this "protagon"-like lipoid of the kidney. From their hydrolysis experiments they came to the conclusion that "carnaubon" represented a triamino-monophosphatide in which an amino-galactose is connected with three fatty acids (carnaubic, stearic and palmitic acid) and with phosphoric acid, whilst the latter is still further connected with two choline groups.

Maclean [1912], in his investigation of the phosphatides of the kidney, obtained a similar lipoid from horse kidney, but he found that the substance contained a water-soluble nitrogenous impurity and that after its removal

¹ Under this name the substance has already passed into the literature. [See Oppenheimer, 1909.]

the nitrogen : phosphorus ratio had fallen from 3 : 1 to 2 : 1. The substance resembled the diamino-phosphatide sphingomyelin in its general properties, but it still furnished a reducing sugar on hydrolysis.

In the course of some unpublished experiments on the products of hydrolysis of brain-"protagon," Rosenheim noticed that the mixture of fatty acids contained one which closely resembled the carnaubic acid described by Dunham as a typical constituent of "carnaubon." Rosenheim found further [1913, 1, 2] that of the constituents of "protagon" the galactoside kerasin yielded this acid (which he identified with lignoceric acid) as its characteristic fatty acid, whilst the phosphatide sphingomyelin also contained it in smaller amounts. Similar results were obtained simultaneously by Levene [1913] and by Thierfelder [1913], who, however, called the acid "kerasinic acid."

In the light of this experience it seemed of interest to reinvestigate the fatty acids of "carnaubon." We prepared therefore this lipid from ox kidneys as well as from horse kidneys according to Dunham and Jacobson's directions and hydrolysed the products by alcoholic sulphuric acid. We obtained in confirmation of Dunham's results a fatty acid of the composition $C_{24}H_{48}O_2$, but we were able definitely to identify this acid with lignoceric acid.

There seems therefore no further justification for the retention of the name "carnaubon" for the "protagon"-like lipid of the kidney¹.

We were unable to find palmitic and stearic acid amongst the products of hydrolysis of "carnaubon," but isolated instead a fatty acid of a higher melting point (94°); this was characterised by its acetyl-derivative as the hydroxy-acid, phrenosinic acid ($C_{25}H_{50}O_3$), which is the fatty acid of the galactoside phrenosin².

Moreover, we found that the basic products of hydrolysis contained not only choline, which Dunham and Jacobson considered as the only base present, but also an ether-soluble base identical with sphingosine, a base also obtained on hydrolysis of brain-"protagon" [Rosenheim and Tebb, 1907].

These facts cannot be made to harmonise with the views of Dunham and Jacobson as to the constitution of "carnaubon." If we consider in conjunction with the above results the fact that the elementary composition of

¹ It may be pointed out in this connection that the uniformity of "carnaubic" acid itself has recently been made doubtful. [Röhmman, 1905, Matthes and Sanders, 1908, Lewkowitsch, 1913.]

² Palmitic and stearic acid were not satisfactorily identified by Dunham and Jacobson (no melting points are given) and their combustion results are in fair agreement with those required by phrenosinic acid, containing an admixture of lignoceric acid.

different preparations of "carnaubon" is extremely variable¹, we come to the conclusion that the kidney-"protagon" represents a variable mixture of lipoids, mainly of galactosides (cerebrosides) and sphingomyelin. In distinction from brain-"protagon" it seems that sphingomyelin preponderates in kidney-"protagon."

EXPERIMENTAL.

"Carnaubon" was prepared from ox kidneys according to Dunham and Jacobson's directions. From 8 kg. fresh ox kidneys were obtained 2.93 g. of twice recrystallised product, which after treatment with water and two further recrystallisations yielded 1.0 g. of carnaubon. In another preparation horse kidneys were employed, about 2 kg. being worked up at a time until 2.5 g. of "pure" carnaubon were available.

Ethyl ester of lignoceric acid.

0.7 g. of carnaubon (ox) was hydrolysed by boiling for six hours under a reflux condenser with 35 cc. absolute alcohol containing 8 % conc. sulphuric acid. The ester crystallised out on cooling in glistening white crystals, which were filtered off on hardened filter paper, washed with cold alcohol and dried in vacuo. The crystals could be easily separated from the paper after drying as a felted silky sheet, which weighed 0.11 g. The ester was twice recrystallised from acetone and finally dried in vacuo at 35°.

It melted at 56° and solidified at 54°. Mixed with lignoceric acid ester from wood paraffin² it showed exactly the same melting and solidifying point.

The quantity of the ester available was sufficient for two micro-combustions.

(1) 13.050 mg.; 37.600 mg. CO₂; 15.175 mg. H₂O.

(2) 8.465 mg.; 24.275 mg. CO₂; 9.840 mg. H₂O.

	(1)	(2)	Calc. for C ₂₂ H ₄₇ COOC ₂ H ₅
C	78.58 %	78.21 %	78.71 %
H	13.01 %	13.01 %	13.22 %

2 g. of carnaubon (horse) were hydrolysed by boiling with 100 cc. absolute alcohol containing 5 % conc. sulphuric acid as before. 0.47 g. of the ester was obtained, which was recrystallised from acetone as above. It showed a

¹ The elementary composition of "carnaubon" obtained by Dunham [1905], Dunham and Jacobson [1910] and Maclean [1912] shows the following variations:

C %	H %	P %	N %	S %
65.61-68.19	11.00-12.37	2.02-3.44	2.55-3.26	0.0-0.82

² Prof. Hell, the discoverer of lignoceric acid [1880], kindly put at Dr Rosenheim's disposal some years ago samples of his original lignoceric acid and its ethyl ester, for which we wish to take this further opportunity of thanking him.

melting point of 56.1° – 56.4° and solidified at 53.6° . A mixture with Hell's ethyl ester of lignoceric acid melted at the same temperature.

Molecular weight estimation. 0.3835 g. was saponified by boiling for three hours with 16.04 cc. 0.1 N alcoholic potash. The excess of potash was then titrated back by means of 0.1 N HCl. 9.71 cc. of 0.1 N KOH had been used up. M.W. = 395. Calc. for $C_{23}H_{47}COOC_2H_5$: 396.

Lignoceric acid.

The potassium salt of the acid was prepared from the ester by saponification with alcoholic potash. The salt was well washed with alcohol and acetone, dried in vacuo and the acid set free by dilute sulphuric acid. The free fatty acid was repeatedly washed with water by fusing its watery suspension on the water-bath. It was then extracted with ether, the ether solution washed with water in a separating funnel, filtered through dry filter paper and evaporated. The white crystalline residue of the free acid was recrystallised from a mixture of acetone and petroleum ether. It was obtained in silky white crystals, which assumed a wax-like consistency when compressed in an agate mortar.

It melted sharply at 81° and solidified at 74.2° – 73.5° . Samples were mixed with Hell's lignoceric acid, further with lignoceric acid prepared from arachis oil [Kreiling, 1888] and with lignoceric acid from kersasin [Rosenheim, 1913, 1, 2].

The melting points of the mixtures are given in the following table.

		Melts at	Solidifies at
Acid from "carnaubon" mixed with	Lignoceric acid (Hell)	81.1°	74.3 – 73.4°
	Lignoceric acid from arachis oil	81.0°	74.5 – 73.5°
	Lignoceric acid from kersasin	81.0°	74.4 – 73.5°

Molecular weight estimation. 0.2192 g. of the acid, dissolved in benzene and petrol ether, was titrated with 0.1 N alcoholic potash (phenolphthalein as indicator) and required 6.00 cc. 0.1 N KOH.

M.W. = 365. Calc. for $C_{24}H_{48}O_2$: 368.

From the potassium salt obtained in the above molecular weight estimation the acid was again set free as before and after a further recrystallisation showed the same melting point (81.1°) as before.

Lead salt.

0.1 g. of the acid, twice purified by means of the potassium salt, was dissolved in methyl alcohol and precipitated with a solution of lead acetate in methyl alcohol. A drop of dilute methyl alcoholic ammonia was added to complete the precipitation. The voluminous white lead salt was filtered, washed with hot acetone and dried in vacuo. It was obtained as a white heavy powder which melted at 117°. Hell and Hermanns [1880] gave the melting point of their lead lignocerate as 117°.

	0.1289 g. gave 0.0412 g. lead sulphate
	Calc. for $(C_{26}H_{47}O_2)_2 Pb$
Pb: 21.83 %	21.99 %

For the purpose of isolating the other products of hydrolysis, the original alcoholic solution (after the filtration of the ester of lignoceric acid) was diluted with water and the alcohol evaporated on the water-bath. An oily semi-solid substance gradually collected on the surface, and solidified on cooling. This substance was found to consist of a mixture of phrenosinic acid (and its ester) and sphingosine sulphate, which were separated by means of ether, in which the sulphate of the base is insoluble.

*Phrenosinic acid*¹. After evaporation of the alcohol the opaque watery fluid was extracted with ether. The ether residue was dissolved in alcohol and neutralised with alcoholic potash. An insoluble potassium salt settled down, from which the fatty acid was set free by dilute sulphuric acid. The free acid was taken up in ether and afterwards recrystallised twice from acetone. It crystallised from alcohol in the characteristic "mamillary" masses which have already been described by Thudichum and later by Thierfelder. The substance melted at 92° and after two recrystallisations at 93°–94°. Levene and Jacobs [1912] have shown that phrenosinic acid exists in two optically isomeric forms, of which the inactive one melts at 82°–85° (Thudichum's neurostearic acid) and the dextro-rotatory form at 106°–108° (Thierfelder's cerebronic acid), whilst a mixture of the two melts at 92°–95°. The acid obtained by us from "carnaubon" is evidently a mixture of the two isomeric forms. It is interesting to note that Grey [1913] also isolated a phrenosinic (cerebronic) acid of the melting point 91° from the mixed fatty acids of brain.

Acetyl-phrenosinic acid. The free acid was boiled for two hours with an excess of acetic anhydride. After cooling, the crystalline acetyl product was

¹ Phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. [See O. Rosenheim, 1914.]

filtered, washed with alcohol and dried in vacuo over soda lime. It was recrystallised from petrol ether and melted at 64° – 65° . It solidified at 62° – 63° . According to Levene and West [1913] the acetyl derivative of the inactive phrenosinic acid "melts at 55.5° – 56° and solidifies at 53° – 54° . The active form would melt higher."

Sphingosine. After the extraction of the phrenosinic acid by ether, the somewhat gelatinous precipitate of sphingosine sulphate left in the aqueous hydrolysis fluid was filtered and washed. The substance was dissolved in alcohol and the base set free by means of alcoholic potash. After dilution with water, it was extracted with ether and remained after removal of the ether as a partly crystalline mass. The free base was converted into the sulphate by neutralising its alcoholic solution with alcoholic sulphuric acid. The insoluble sulphate was filtered off and recrystallised twice from alcohol. It crystallised in needles arranged in rosettes and was finally obtained as a white crystalline powder. On heating it gradually decomposes and melts at 243° – 244° , effervescing at 250° . Thierfelder [1904] states that it decomposes with effervescence between 240° – 250° . The substance gives with sulphuric acid and sucrose a typical Pettenkofer's reaction and yields nitrogen with nitrous acid. A quantitative estimation in van Slyke's micro-apparatus was unfortunately lost owing to unexpected excessive frothing.

The final aqueous hydrolysis fluid gave Molisch's test and contained a reducing sugar (galactose?) and phosphoric acid. The presence of choline was demonstrated by the periodide test.

We are at present engaged with the preparation of larger quantities of the material, in order to separate the galactosides from sphingomyelin by the pyridine method [Rosenheim, 1914] and to investigate further their cleavage products.

The expenses of this research have been in part defrayed from a grant to Dr O. Rosenheim from the Government Grant Committee of the Royal Society.

SUMMARY.

1. The fatty acid $C_{24}H_{48}O_2$ obtainable on hydrolysis of "carnaubon" is not carnaubic acid, but is identical with lignoceric acid.
2. Phrenosinic acid ($C_{25}H_{50}O_3$) and the base sphingosine ($C_{17}H_{35}NO_2$) were isolated as hitherto unrecognised cleavage products of "carnaubon."
3. There is no justification for the retention of the name "carnaubon" for the lipid mixture which can be obtained from kidneys by extraction with alcohol.

REFERENCES.

- Dunham, E. K. (1905). *Proc. Soc. Exp. Biol. Med.*, **2**, 61.
— (1908). *J. Biol. Chem.*, **4**, 297.
— and Jacobson, C. A. (1910). *Zeitsch. physiol. Chem.*, **64**, 302
Grey, E. C. (1913). *Biochem. J.*, **7**, 148.
Hell, C. (1880). *Ber.*, **13**, 1709.
— and Hermanns, O. (1880). *Ber.*, **13**, 1713.
Kreiling, B. (1888). *Ber.*, **21**, 880.
Levene, P. A. (1913). *J. Biol. Chem.*, **15**, 359.
— and Jacobs, W. A. (1912). *J. Biol. Chem.*, **12**, 381.
— and West, C. J. (1913). *J. Biol. Chem.*, **14**, 257
Lewkowitsch, J. (1913). *Oils, Fats and Waxes*, **1**, 168.
Maclean, H. (1912). *Biochem. J.*, **6**, 333.
Matthes, H. and Sanders, H. (1908). *Arch. Pharm.*, **246**, 165.
Oppenheimer (1909). *Handbuch der Biochemie*, **1**, 145
Röhmman, F. (1905). *Centrl. Physiol.*, **19**, 317.
Rosenheim, O. (1913, 1). *Trans. Internat. Congr. Med.*, Sect. II., 626.
— (1913, 2). *Biochem. J.*, **7**, 604.
— (1914). *Biochem. J.*, **8**, 110.
— and Tebb, M. C. (1907). *J. Physiol.*, **36**, 1.
Thierfelder, H. (1904). *Zeitsch. physiol. Chem.*, **43**, 21.
— (1913). *Zeitsch. physiol. Chem.*, **85**, 35.