XV. THE GALACTOSIDES OF THE BRAIN. IV. THE CONSTITUTION OF PHRENOSIN AND KERASIN.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

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In the first communication of this series [1913, 2] I expressed the view that the brain contains in a preformed condition mainly two representatives of the group of glucosides, namely the galactosides phrenosin and kerasin, which were originally discovered by Thudichum. A new method for their preparation was subsequently described together with a physical test (the selenite plate test) which furnishes a reliable method for controlling the efficacy of the methods used for their separation [1914, 1].

In the following communication are given the experimental data obtained by the chemical and physical examination of the galactosides together with the results of complete hydrolysis experiments.

The outcome of these experiments has been to establish for the first time the fact that kerasin possesses a laevorotation in distinction from the dextro-rotatory phrenosin, and further that the only difference in chemical constitution of the two glucosides consists in their fatty acid component¹.

Whilst kerasin furnishes on hydrolysis inactive lignoceric acid ($C_{24}H_{48}O_2$), phrenosin gives rise to the optically active hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$). The near relationship of these fatty acids is evident from the interesting fact that phrenosinic acid furnishes lignoceric acid on oxidation [Levene and Jacobs, 1912, 2]. Both glucosides yield the same carbohydrate, *d*-galactose, and the same base, sphingosine ($C_{17}H_{35}O_2N$), on hydrolysis.

These observations, together with the facts established by Levene and others with regard to the constitution of sphingosine, enable us now to construct constitutional formulae for these substances.

¹ The results of this work were communicated to the section of physiology of the 17th Internat. Congress of Medicine on Aug. 11th, 1913 [Rosenheim, 1913, 1]. Their publication in full has been delayed owing to unforeseen circumstances.

The unsaturated base sphingosine contains two free hydroxyl groups besides one amino-group [Levene and Jacobs, 1912, 1; Thierfelder and Thomas, 1912; Levene and West, 1914], and its formula may be written as: $C_{12}H_{25}CH:CH \cdot CH(OH) \cdot CH(OH) \cdot CH_2NH_2$. It is evident that in the galactosides the amino-group of the sphingosine is no longer free, since kerasin does not yield any nitrogen with nitrous acid when tested in van Slyke's apparatus. I was also unable to obtain any nitrogen from phrenosin by heating with hypobromite of potassium, and Levene and Jacobs [1912, 3] record incidentally a similar experience when testing their cerebrins in van Slyke's apparatus. We may therefore assume that in the galactosides the NH_2 group of sphingosine is in combination with the carboxylic group of the fatty acids, especially since the substances show neither any acid nor alkaline tendencies¹. The possibility of a linkage of sphingosine by means of the NH_2 group with galactose must not be lost sight of. Up till now, however, no naturally occurring glucosides have been met with in which the carbohydrate is linked to nitrogen, and it is therefore much more likely that the aldehyde group of the galactose enters into combination with one of the hydroxyl groups of sphingosine. In analogy to the constitution of the other known naturally occurring glucosides, we may further conclude that phrenosin and kerasin are β -galactosides, in which galactose possesses the closed chain formation. From these considerations we arrive at the following constitutional formula of kerasin ($C_{47}H_{91}O_8N$):

This formula is in agreement with all the known facts of the reactions and cleavage products of kerasin. It allows for the formation of galactose, lignoceric acid and a salt of sphingosine during hydrolysis with a mineral acid in aqueous solution, whilst it also explains the formation of mono-methyl sphingosine and the methyl ester of lignoceric acid during methyl alcoholysis (see later). It contains five free hydroxyl groups which is in accordance with the formation of a penta-acetyl kerasin, a derivative prepared by Thierfelder [1914]. The position of the unoccupied hydroxyl group in the sphingosine residue in the above formula is arbitrary, since the relative position of the hydroxyl groups in sphingosine itself is still unknown.

¹ The tentatively advanced formulae [Rosenheim, 1913, 1] in which the presence of a free NH_2 group is assumed require therefore a modification in this direction.

The constitution of phrenosin is analogous to that of kersin, the hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$) taking the place of lignoceric acid. This is expressed by the following constitutional formula of phrenosin ($C_{48}H_{93}O_9N$):

We must assume that in the phrenosin molecule the two hydroxyl groups of sphingosine are at all events partially combined with other groups, since on alcoholysis besides sphingosine a certain amount of dialkyl sphingosine is formed. It is most probable that partial anhydride formation takes place between the sphingosine and phrenosinic acid hydroxyls. It is not likely that one of the hydroxyl groups of the galactose molecule is thus linked up, since on this assumption one would expect to find at least an indication of the formation of an alkyl galactose on alcoholysis. The linkage between the hydroxyl groups of phrenosinic acid and sphingosine is evidently only partial, since phrenosin furnishes on acetylation a hexa-acetyl derivative [Thierfelder, 1914]. This assumption also allows a satisfactory explanation for the existence of the two modifications of phrenosin. The ordinary "amorphous" phrenosin, which easily assumes the liquid-crystalline form [Rosenheim, 1914, 2], corresponds to the anhydric formula, whilst in the crystallised phrenosin the two hydroxyl groups are free. In accordance with this view is the fact that the crystallised modification contains one molecule of water more than the amorphous modification [Rosenheim, 1914, 2; see also Thierfelder and Wörner, 1900]. If this view is correct, the completely crystallised modification should give rise to a monoalkyl sphingosine on alcoholysis, a possibility which I have not yet tested experimentally. It may, however, be noted in this direction that Thierfelder and Thomas [1912] obtained on methyl alcoholysis of a sample of "cerebron" a substance which they considered at first as a monomethyl sphingosine. As the "cerebron" employed in this experiment consisted of a mixture of the crystallised and amorphous modifications, it is not unlikely that the cleavage product was a mixture of sphingosine with monomethyl sphingosine.

It is to be expected that the study of the products of a partial hydrolysis of these galactosides will furnish conclusive evidence to decide the correctness of the constitutional formulae advanced above, which may serve in any case as a basis for further research. Thudichum has already indicated the existence of two such compounds, which he called aesthesin (phrenosin minus galactose) and psychosin (phrenosin minus phrenosinic acid). The author is at present engaged in studying the action of enzymes on these galactosides. It is hoped that a suitable lipoclastic enzyme may split off the fatty acid component, leaving a sphingosine-galactoside, whilst an enzyme of the emulsin type may split off galactose and leave the sphingosine-fatty acid combination intact. The experiments made up till now show that phrenosin is resistant to pancreatic lipase and emulsin (commercial). An extract of brain from a case of extensive brain softening also had no action on phrenosin.

EXPERIMENTAL.

I. *Phrenosin*.

Elementary analysis. The analyses (1) and (2) refer to different samples of amorphous phrenosin prepared by the new pyridine method [Rosenheim, 1914, 1]. Analysis (3) was made of a sample of crystallised phrenosin [Rosenheim, 1914, 2]. The substances were dried at 105°.

- (1) 0.1244 g.; 0.3183 g. CO₂; 0.1293 g. H₂O,
0.1293 g.; 2.3 cc. N at 26.0° and 714.5 mm.
- (2) 0.1255 g.; 0.3207 g. CO₂; 0.1267 g. H₂O,
0.1321 g.; 2.2 cc. N at 26.0° and 714.5 mm.
- (3) 0.1300 g.; 0.3305 g. CO₂; 0.1291 g. H₂O,
0.0960 g.; 1.7 cc. N at 17.5° and 722.5 mm.

	(1)	(2)	(3)	Average	Calc. for C ₄₈ H ₈₈ O ₉ N
C	69.78 %	69.69 %	69.34 %	69.60 %	69.65 %
H	11.63 „	11.29 „	11.11 „	11.34 „	11.24 „
N	1.90 „	1.79 „	1.91 „	1.83 „	1.70 „

Melting point. Phrenosin on heating assumes the liquid-crystalline condition, from a temperature of about 95° upwards until 210–212°, when it changes into the amorphous liquid condition. These phenomena have been fully described in a previous communication [1914, 2]. Phrenosin prepared by Thudichum's original method, as well as by that of Thierfelder, shows the same behaviour as phrenosin prepared by the pyridine method.

Optical activity. Phrenosin is dextrorotatory and the degree of its rotation is greatly influenced by the nature of the solvent, temperature and concentra-

tion. Pyridine is the most convenient solvent at ordinary temperatures and was first used in 1907 for this purpose by Rosenheim and Tebb in connection with some other work on brain lipoids. A 10 % solution of phrenosin in pyridine, however, possesses at 20° only about half the optical activity of a 5 % solution in chloroform-methyl alcohol at 40°, whilst a 10 % solution in the latter solvent at 45° shows nearly three times the optical activity of a 10 % pyridine solution at 20°. The estimations were made in a large Lippich polarimeter in a 1 dm. tube in sodium light.

Phrenosin (Pyridine method).

- (1) 1.0046 g. in 10 cc. pyridine; $t = 20^\circ$; $\alpha = +0.38^\circ$; $[\alpha]_D^{20^\circ} = +3.78^\circ$.
 - (2) 1.0011 g. in 10 cc. pyridine; $t = 20^\circ$; $\alpha = +0.37^\circ$; $[\alpha]_D^{20^\circ} = +3.70^\circ$.
- 1.0000 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 45^\circ$; $\alpha = +1.04^\circ$; $[\alpha]_D^{45^\circ} = +10.4^\circ$.

Phrenosin (Thudichum's method).

- 1.000 g. in 10 cc. pyridine; $t = 18^\circ$; $\alpha = +0.37^\circ$; $[\alpha]_D^{18^\circ} = +3.70^\circ$.
- 1.000 g. in 10 cc. pyridine; $t = 30^\circ$; $\alpha = +0.43^\circ$; $[\alpha]_D^{30^\circ} = +4.30^\circ$.
- 0.500 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 40^\circ$; $\alpha = +0.37^\circ$; $[\alpha]_D^{40^\circ} = +7.40^\circ$.

Thierfelder and Kitagawa [1906] have previously found for "cerebron" $[\alpha]_D^{50^\circ} = +6.4$ to $+8.4^\circ$ in 5 % chloroform-methyl alcohol and Levene and Jacobs. [1912, 3] for "*d*-cerebrin" (prepared by the baryta method) $[\alpha]_D^{25^\circ} = +1.01$ to $+1.88^\circ$ in pyridine. Later Levene [1913] has examined "cerebrin" fractions obtained by a process not yet fully described, for which he found $[\alpha]_D = +3.05$ to $+4.14^\circ$ in pyridine and $[\alpha]_D = +9.5$ to $+10.7^\circ$ in 6 % chloroform-methyl alcohol. My results agree, therefore, both with the earlier ones of Thierfelder and the later ones of Levene.

Hydrolysis. Many samples of phrenosin, prepared both by Thudichum's original method and by the pyridine method were hydrolysed. The products of hydrolysis were identical in both cases, qualitatively as well as quantitatively, namely (1) phrenosinic acid, (2) sphingosine and (3) galactose. Hydrolysis was effected by boiling under ordinary pressure (*a*) with dilute aqueous hydrochloric acid, or (*b*) with methyl alcoholic sulphuric acid. In the latter case the methyl-ester of phrenosinic acid and small quantities of dimethylsphingosine, in addition to sphingosine, are formed.

- (1) *Phrenosinic acid* ($C_{25}H_{50}O_3$)¹. 1.0 g. phrenosin (prepared by the

¹ Phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. (See O. Rosenheim, 1913, 2.)

pyridine method) was hydrolysed by boiling its solution in 50 cc. methyl alcohol, containing 5 % sulphuric acid, for 5.5 hours on a reflux. The solution remained perfectly colourless and on cooling a crystallised white precipitate formed. The mixture of free fatty acid and ester was filtered off, washed with cold methyl alcohol and converted into the sodium salt by means of sodium methylate in methyl alcoholic solution. The sodium salt was recrystallised from boiling absolute alcohol and the acid liberated by dilute sulphuric acid. The free acid was taken up in ether and its ethereal solution washed with water in a separating funnel until free from mineral acid. The white fatty acid left on evaporation of the filtered ether solution weighed 0.305 g. and fused at 103°–104°.

Phrenosinic acid crystallises from alcohol in characteristic forms which Thudichum [1881] has described as “mamillary” or cauliflower-like masses. Under the polarising microscope they are seen to consist of a conglomerate of irregular spherocrystals, which behave in the selenite plate test [Rosenheim, 1914, 1] exactly as the mother substance phrenosin.

For analysis the acid was recrystallised three times from acetone. After the last recrystallisation and drying *in vacuo* it showed a melting point of 105°–106° and a dextrorotation of $[\alpha]_D^{20} = +3.86^\circ$ in pyridine ($l = 1$; $c = 2.849$; $t = 20^\circ$; $a = +0.11^\circ$).

On analysis it gave the following figures:

0.0792 g.; 0.2195 g. CO₂; 0.0894 g. H₂O.

	Found	Calc. for C ₂₅ H ₅₀ O ₃
C	75.58 %	75.30 %
H	12.63 „	12.65 „

Sodium salt. Another preparation of phrenosinic acid was converted into the sodium salt, which was recrystallised from absolute alcohol and dried at 105°. 0.1042 g. gave 0.0172 g. Na₂SO₄;

Na = 5.39 %. Calculated for C₂₅H₄₉O₃Na = 5.48 %.

Molecular weight estimation. A sample of phrenosin, prepared by Thudichum's method, was hydrolysed as above described. The ester was converted into the potassium salt and the free acid, prepared from it, recrystallised from acetone. It fused at 102°–103°. The acid was dissolved in benzene and titrated with 0.1N alcoholic potash (phenolphthalein as indicator). 0.3257 g. required 8.24 cc. 0.1N KOH.

Molecular weight = 395. Calc. for C₂₅H₅₀O₃: 398.

Another sample was hydrolysed by boiling for 24 hours with dilute hydrochloric acid. The mixture of free fatty acid and sphingosine hydrochloride was filtered after the addition of a few cc. of saturated sodium sulphate solution which renders quick filtration possible. The acid was extracted with ether and purified by means of the barium salt. The barium salt was decomposed with tartaric acid and the free acid extracted with ether. It fused at 102° – 103° . 0.2687 g. required 6.73 cc. 0.1N KOH. Molecular weight = 399. Calc. for $C_{25}H_{50}O_3$: 398.

Melting point of phrenosinic acid. The fatty acid obtained by Thudichum from phrenosin and called by him "neurostearic" acid had a melting point of 84° , but the same elementary composition as phrenosinic acid. Thierfelder's "cerebronic" acid, prepared by a method almost identical with that of Thudichum, melted at 98° – 99° but yielded on analysis practically the same figures as Thudichum's acid. Mainly on account of the difference in their melting points, Thierfelder [1905] declined to admit the identity of these acids and their mother substances. More recently, however, Levene and Jacobs [1912, 2] again obtained Thudichum's acid of the lower melting point and showed that "cerebronic" acid exists in two optically isomeric forms of which the inactive one melts at 82° – 85° (Thudichum's "neurostearic" acid), whilst the dextrorotatory form melts at 106° – 108° (Thierfelder's "cerebronic" acid). A mixture of the two forms melting at 92° – 95° is frequently obtained [Rosenheim and MacLean, 1915]. The conditions under which these two isomers are formed are not yet explained. In some preliminary experiments I have found that the melting point of the dextrorotatory acid (103° – 104°) is lowered considerably by keeping it for some time in a toluene bath. After six hours the melting point had fallen to 88° – 89° . Experiments are in progress to decide whether this decrease goes hand in hand with a racemisation. The change in melting point may possibly be due also to the formation of an anhydride or lactone-like derivative owing to the presence of a hydroxyl group in phrenosinic acid. The difficulty mentioned by Levene and West [1913] of obtaining correct titration values of the inactive acid is in favour of such an explanation.

(2) *Sphingosine* ($C_{17}H_{35}O_2N$). This base was first obtained by Thudichum on hydrolysis of phrenosin with dilute sulphuric acid under pressure. The peculiar name was given to it "in commemoration of the many enigmas which it presented to the enquirer" [Thudichum, 1881]. Thierfelder [1904] confirmed Thudichum's results when he hydrolysed his "cerebron" by the same method; but later Thierfelder and Kitagawa [1906] expressed doubts

as to the uniformity of sphingosine, as they obtained on hydrolysis in methyl alcoholic solution a mixture of sphingosine with another base of the composition $C_{19}H_{39}O_2N$. These doubts were removed by Levene and Jacobs [1912, 1], who showed that the second base, dimethylsphingosine, was formed during alcoholysis and was not originally present in the phrenosin molecule. These observations were confirmed by Thierfelder and Riesser [1912].

I have obtained sphingosine from phrenosin (prepared by Thudichum's method) on hydrolysis with dilute hydrochloric acid, and both sphingosine and dimethylsphingosine from phrenosin (prepared by the pyridine method) on hydrolysis with sulphuric acid in methyl alcoholic solution. Only the identification of the latter need be described here.

The filtrate obtained above after the removal of phrenosinic acid and its ester was diluted with 100 cc. water and the alcohol evaporated on a water-bath. The solution became cloudy and a semi-solid white substance collected on the surface, which solidified on cooling. This was filtered off and dissolved on the filter in boiling alcohol. On evaporation of the alcohol and drying *in vacuo* the residue of sphingosine sulphate and dimethylsphingosine sulphate weighed 0.550 g. The two substances were separated by their different solubilities in alcohol.

(a) *Sphingosine*. The mixture of the sulphates was treated repeatedly at room temperature with alcohol. From the insoluble portion the base was set free by means of alcoholic potash. It was extracted with ether and the ether solution washed with water. On evaporation of the ether the base remained as a syrup, which crystallised completely in rosettes of needles. The base was dissolved in alcohol and reconverted into the sulphate by the addition of alcoholic sulphuric acid. The process of purification was repeated once more and sphingosine sulphate was obtained in perfectly white crystals, which were filtered, washed with ether and dried *in vacuo*. The salt is hygroscopic and melts with decomposition at 233° – 235° (Levene and Jacobs give 233° – 239°). Its solution in methyl alcoholic sulphuric acid showed a laevorotation of $[\alpha]_D^{18^{\circ}} = -9.47^{\circ}$.

$$(l = 1; \quad c = 1.585; \quad t = 18^{\circ}; \quad a = -0.15^{\circ}.)$$

(b) *Dimethylsphingosine*. The easily soluble sulphate of dimethylsphingosine obtained above was converted into the free base and extracted with ether. The base crystallised at once on evaporation of the ether. It was converted into the hydrochloride by adding a methyl alcoholic solution of hydrochloric acid to its solution in methyl alcohol. The hydrochloride was

recrystallised from acetone, from which it separated in glistening plates. It melted at 133°–134° and gave the following results on analysis:

5.385 mg.; 12.875 mg. CO₂; 5.84 mg. H₂O.

	Found	Calc. for C ₁₈ H ₃₀ O ₈ N · HCl
C	65.20 %	65.18 %
H	11.58 „	11.52 „

(3) *d*-Galactose. The solution from which phrenosinic acid and sphingosine had been removed, as above described, contained only *d*-galactose as the last product of hydrolysis. This was estimated quantitatively by means of the polarimeter. The solution was made up to 100 cc. and showed in a 2 dm. tube a rotation of $\alpha = +0.35^\circ$. Whence galactose = 21.63 %; calc. for 1 mol. galactose from C₄₈H₉₈O₉N 21.77: %.

d-Galactose was identified by means of its characteristic methylphenylhydrazone. Solid sodium acetate was added until the reaction was no longer acid to Congo paper. A few drops of methylphenylhydrazine were added, and after standing at room temperature crystals of the hydrazone settled down. They were filtered off, washed with water and recrystallised from alcohol. The perfectly white crystals of the hydrazone melted sharply at 190°. Neuberg [1907] gives the melting point of galactose methylphenylhydrazone at 191°.

Comparison of the galactoside, prepared from brain by the pyridine method, with Thudichum's phrenosin shows their complete agreement in all points. It follows from this that Thierfelder's "cerebron," with which my substance agrees in every way, is also identical with phrenosin. Gamgee's "pseudo-cerebrin" is admittedly identical with "cerebron" prepared by Thierfelder's original method (1904) whilst there can be no doubt that the "cerebrin" of Parcus as well as that of Kossel and Freytag is identical with "cerebron" prepared by Thierfelder's later baryta method [1913].

Hence it follows that all these products represent one and the same substance, namely phrenosin. The unfortunate confusion which has been brought into the nomenclature of the brain lipoids by the introduction of new names for one and the same substance can now be considered to be cleared away experimentally. It seems only just to retain the name phrenosin introduced in 1874 by Thudichum, who discovered the substance and was the first to establish the nature of its cleavage products. This suggestion has previously been made, based on theoretical considerations, by Posner

and Gies [1906] and Rosenheim and Tebb [1907] and was adopted by myself in the proposal for the nomenclature of the lipoids [1909].

II. Kerasin.

Elementary analysis. The analyses (1)–(4) were made of different samples of kerasin prepared by the new pyridine method and sample (5) refers to kerasin prepared by Thudichum's original method, slightly modified [Rosenheim, 1914, 1]. The substances were dried *in vacuo* over sulphuric acid.

- (1) 0.1363 g.; 0.3444 g. CO₂; 0.1395 g. H₂O,
0.1416 g.; 2.4 cc. N at 24° C. and 714 mm.
- (2) 0.1317 g.; 0.3331 g. CO₂; 0.1372 g. H₂O.
- (3) 0.1200 g.; 0.3034 g. CO₂; 0.1205 g. H₂O,
0.1379 g.; 2.3 cc. N at 25° C. and 714.5 mm
- (4) 0.1185 g.; 0.2997 g. CO₂; 0.1187 g. H₂O,
0.1285 g.; 2.2 cc. N at 21° C. and 716 mm.
- (5) 0.1156 g.; 0.2920 g. CO₂; 0.1184 g. H₂O,
0.1578 g.; 2.6 cc. N at 22.5° C. and 710 mm.

	(1)	(2)	(3)	(4)	(5)	Calc. for C ₄₇ H ₉₁ O ₈ N + H ₂ O
C	68.91	68.98	68.95	68.98	68.90	69.14 %
H	11.44	11.65	11.23	11.21	11.46	11.49 „
N	1.83	—	1.74	1.86	1.76	1.72 „

The values obtained are somewhat lower than those previously recorded in the literature. This may be due to the fact that the substances were dried *in vacuo* at ordinary temperature instead of at 100°–105°. The figures agree well with the theoretical formula C₄₇H₉₁O₈N + H₂O which is calculated on the assumption that the kerasin molecule is composed of lignoceric acid (C₂₄H₄₈O₂), sphingosine (C₁₇H₃₅O₂N) and galactose (C₆H₁₂O₆) in equimolecular proportions.

It may be pointed out again that the elementary analysis is an unreliable index of the purity of these complex galactosides and does not prove the absence of phrenosin in kerasin preparations and *vice versa*. The difference in elementary composition between phrenosin (C₄₈H₉₃O₉N) and kerasin (C₄₇H₉₁O₈N) is so small that the presence of even a large percentage of phrenosin cannot be detected by elementary analysis. This is well illustrated by the experience of Levene and Jacobs [1912, 3], who originally considered phrenosin and kerasin as optical isomeric substances of the same chemical

composition, a view which seemed to receive support by the agreement of their elementary analyses. Further Thierfelder's [1913] substances of the kerasin type also yielded analytical figures which agreed well with the calculated formula and still it was found on hydrolysis that 25 % of the fatty acids consisted of the acid $C_{25}H_{50}O_2$, which is the characteristic component of phrenosin.

It is clear, therefore, that elementary analysis of these highly complex substances is of little value. Up till now the only satisfactory criteria of their purity are furnished by an examination of their optical activity and by the selenite test [Rosenheim, 1914, 1].

Melting point. The behaviour of kerasin on heating and the formation of liquid crystals has been fully described in a previous communication.

Optical activity. Kerasin is laevorotatory in distinction from the dextro-rotatory phrenosin and the degree of its laevorotation may serve as an index for the more or less successful removal of phrenosin. Its optical activity increases with rising temperature and varies with the concentration and the nature of the solvent. The most highly purified specimens so far obtained showed an optical activity in pyridine at room temperature of $[\alpha]_D = -3.71$ to -3.78° . In a solution of chloroform containing 10 % pyridine, at 50° , their activity was $[\alpha]_D^{50^\circ} = -4.58$ to -5.08° .

- (1) 0.9132 g. in 10 cc. pyridine:

$$t = 20^\circ; \quad l = 1; \quad \alpha = -0.25^\circ; \quad [\alpha]_D^{20^\circ} = -2.74^\circ.$$

- (2) 0.9004 g. in 10 cc. pyridine:

$$t = 18^\circ; \quad l = 1; \quad \alpha = -0.25^\circ; \quad [\alpha]_D^{18^\circ} = -2.78^\circ.$$

- (3) 1.0000 g. in 10 cc. pyridine:

$$t = 18^\circ; \quad l = 1; \quad \alpha = -0.25^\circ; \quad [\alpha]_D^{18^\circ} = -2.50^\circ.$$

- (4) 1.0501 g. in 10 cc. pyridine:

$$t = 25^\circ; \quad l = 1; \quad \alpha = -0.39^\circ; \quad [\alpha]_D^{25^\circ} = -3.71^\circ.$$

1.0044 g. in 10 cc. chloroform containing 10 % pyridine:

$$t = 50^\circ; \quad l = 1; \quad \alpha = -0.51^\circ; \quad [\alpha]_D^{50^\circ} = -5.08^\circ.$$

Kerasin (Thudichum's method)¹.

1.0052 g. in 10 cc. pyridine:

$$t = 25^\circ; \quad l = 1; \quad \alpha = -0.38^\circ; \quad [\alpha]_D^{25^\circ} = -3.78^\circ.$$

0.5025 g. in 10 cc. pyridine-acetone (1 : 1):

$$t = 50^\circ; \quad l = 1; \quad \alpha = -0.23^\circ; \quad [\alpha]_D^{50^\circ} = -4.58^\circ.$$

¹ In connection with some work on the optical activity of brain lipoids Rosenheim and Tebb [1907] examined a sample of kerasin prepared by Thudichum's method and found its optical activity in a 3 % pyridine solution to be $[\alpha]_D^{18^\circ} = -2.8^\circ$.

The kerasin fractions examined by Levene and Jacobs [1912, 3] were found by them to be inactive and they therefore proposed for kerasin the name *dl*-cerebrin (phrenosin = *d*-cerebrin). It may be pointed out, however, that they note that "once a sample was obtained from the kerasine fraction that was strongly laevorotatory." Thierfelder's [1913] kerasin fractions were also inactive or even slightly dextrorotatory. It is obvious from the above-mentioned presence of phrenosinic acid in them that they were contaminated with the dextrorotatory phrenosin to the extent of at least 12 %.

Molecular weight. Only one attempt has up till now been made to determine the molecular weight of kerasin by a physical method. Kossel and Freytag [1893] calculate from the rise of boiling point in glacial acetic acid solution the molecular weight of "homocerebrin" (= kerasin) as 986 (945, 1027). These figures can only be taken as approximate since it is well known that the accuracy of Beckmann's method is considerably impaired when applied to substances of high molecular weight which tend to form colloidal solutions. This may also be inferred by the experience of Pearson [1914]¹. Yet it seemed worth while to attempt the estimation of the molecular weight of kerasin by using Barger's [1904] microscopical method based on vapour pressure. Dr A. J. Ewins, who possesses considerable experience with Barger's method, kindly carried out the observations, for which I wish to take this opportunity of thanking him. Employing pyridine as a solvent and benzil as a standard, a solution of 0.080 g. kerasin in 0.500 g. of solvent was found to be between 0.20 and 0.22 mol., *i.e.* molecular weight lies between

$$\frac{0.080 \times 1000}{0.500 \times 0.20} = 800 \text{ and } \frac{0.080 \times 1000}{0.500 \times 0.22} = 727. \text{ Mean value} = 763.$$

¹ A chloroform solution of a mixture of galactosides, prepared by the baryta process, showed an elevation of the boiling point which stands in no relationship to the molecular weight. Pearson draws from his observations the conclusion that the galactosides occur in a chemical combination with sphingomyelin in the so-called "protagon." A single sample of "protagon," of which not even an analysis is given, was examined and failed to show any elevation of the boiling point, whilst a mixture of galactosides and sphingomyelin (prepared from "protagon" by the pyridine method) produced a measurable elevation. Quite apart from the probability that a similar measurable elevation might be found if several samples of "protagon" had been examined, the results recorded are to be expected, if only two components of the complex mixture "protagon" are examined by this method. The author failed to reconstitute the mixture "protagon" in its entirety, since he omitted to add the colloidal substances always present in protagon. These were removed by baryta in the one case and by the pyridine treatment in the other. It is admitted by Pearson that the contentions which were made in favour of the chemical entity of "protagon" were inconclusive and the whole case for "protagon" is now reduced to the value which can be attached to the few incomplete observations quoted. On the other hand the clear and experimentally uncontradicted chemical evidence has established the fact that "protagon" is a heterogeneous mixture.

Considering the size of the molecule, this figure agrees fairly well with the molecular weight ($C_{47}H_{91}O_8N = 797$) as calculated from the cleavage products of kerašin (see above).

It is interesting to note that in the case of phrenosin the method failed owing to the colloidal condition of sufficiently concentrated solutions.

Hydrolysis. This was carried out by the same methods as those employed for phrenosin previously described. Samples of kerašin prepared by the pyridine method as well as by Thudichum's method were hydrolysed and gave identical results. The products of hydrolysis were: (1) lignoceric acid, $C_{24}H_{48}O_2$; (2) sphingosine; and (3) galactose.

(1) *Lignoceric acid.* (a) *Methyl ester.* 3.50 g. of kerašin were hydrolysed by boiling under a reflux condenser for 6 hours with 250 cc. methyl alcohol containing 10 % conc. H_2SO_4 . On cooling glistening white crystals of the methyl ester of lignoceric acid crystallised out which were filtered and dissolved in a small quantity of methyl alcohol. In order to complete the esterification HCl gas was passed into the hot solution and the ester, which crystallised out on cooling, was filtered, washed and dried *in vacuo*. It weighed 1.27 g. and was recrystallised in two fractions from acetone. The two fractions showed the same melting point of 57° – 58° . According to Hell and Hermanns [1880] the methyl ester of lignoceric acid from wood paraffin melts at 56.5° – 57° and Kreiling [1888] found the melting point 58° for the methyl ester of lignoceric acid from arachis oil.

The two fractions were combined and again recrystallised from acetone. The melting point remained unaltered. For elementary analysis the substance was dried *in vacuo* at 35° .

(9.075 mg.; 26.1155 mg. CO_2 ; 10.910 mg. H_2O .)

	Found	Calc. for $C_{23}H_{47}COOCH_3$
C	78.58 %	78.44 %
H	13.44 „	13.18 „

Molecular weight. 0.6044 g. of the ester was saponified by boiling for three hours with alcohol containing 20.42 cc. 0.1N KOH. The excess of potash was then titrated back by means of 0.1N HCl. 16.01 cc. of 0.1N KOH had been used up. Molecular weight = 382. Calc. for $C_{23}H_{47}COOCH_3$: 382.

Another sample of kerašin was hydrolysed in ethyl alcoholic solution. The ethyl ester of lignoceric acid was obtained and melted at 56° , solidifying

at 54°. Mixed with lignoceric acid ethyl ester from wood paraffin¹, it showed exactly the same melting and solidifying points.

(b) *Free lignoceric acid*. The potassium salt of lignoceric acid was obtained by saponification of the methyl and ethyl esters, filtered and well washed with alcohol and acetone. The fatty acid was set free from the soap by means of dilute hydrochloric acid and freed from mineral acid by repeatedly fusing its watery suspension on the water-bath. It was then extracted with ether, its ethereal solution washed with water, filtered through dry filter paper and evaporated. The white crystalline free acid was dried *in vacuo* and showed a melting point of 81°.

The acid was recrystallised twice from a mixture of light petroleum and acetone and was obtained in silky white crystals, forming a loose powder which assumed a wax-like consistency when compressed in an agate mortar.

Melting point. The substance had a sharp melting point of 81.0° and solidified at 74.5°–73.5°. The melting point of lignoceric acid is given in the literature as 80.5°–81° [Hell and Hermanns, 1880; Kreiling, 1888]. Samples were mixed with Hell's lignoceric acid and with lignoceric acid prepared by myself from arachis oil according to Kreiling. The melting points of the various mixtures were identical at 81.0°.

Optical activity. A 10 % solution of the acid in chloroform at 25° was found to be inactive.

Elementary analysis.

(1)	0.0745 g.;	0.2136 g. CO ₂ ;	0.0862 g. H ₂ O.
(2)	5.145 mg.;	14.810 mg. CO ₂ ;	5.848 mg. H ₂ O.
	(1)	(2)	Calc. for C ₂₄ H ₄₈ O ₂
C	78.19 %	78.50 %	78.17 %
H	12.95 „	12.72 „	13.13 „

Molecular weight. (a) 0.2000 g. of the acid was titrated in light petroleum solution with 0.1N alcoholic potash (phenolphthalein as indicator) and required 5.39 cc. 0.1N KOH.

(b) 0.2205 g. of a different preparation (Thudichum's) required 5.97 cc. 0.1N KOH.

	(a)	(b)	Calc. for C ₂₄ H ₄₈ O ₂
Molecular weight	371	369	368

¹ Through the kindness of Prof. Hell, the discoverer of lignoceric acid [1880], I came into the possession of samples of his original lignoceric acid and its ethyl ester, which also served for the identification of lignoceric acid from kidney lipoids [Rosenheim and MacLean, 1915].

The acid recovered from the potassium salts after titration showed the same melting point as before.

Silver salt. The acid was dissolved in alcohol and the silver salt precipitated by means of an alcoholic solution of silver nitrate. A drop of dilute alcoholic ammonia was added and the silver salt, which is not very sensitive to light, was filtered, washed with boiling alcohol and dried in a dark desiccator.

0.1208 g. gave 0.0273 g. Ag = 22.59 %,

Calc. for $C_{23}H_{47}COOAg$: 22.74 %.

Lead salt. The acid was dissolved in methyl alcohol and precipitated with a solution of lead acetate in methyl alcohol. A small amount of dilute methyl alcoholic ammonia was added to complete the precipitation. The lead salt was filtered, washed with hot acetone and dried *in vacuo*. It formed a white heavy powder which melted at 117°. The melting point of lead lignocerate is given as 117° by Hell and Hermanns [1880]. Another preparation was made from lignoceric acid obtained by the hydrolysis of Thudichum's kersin.

(1) 0.1804 g. gave 0.0577 g. lead sulphate.

(2) 0.1508 g. gave 0.0482 g. lead sulphate.

	Pb	Calc. for $(C_{24}H_{47}O_2)_2Pb$
(1)	21.84 %	
(2)	21.83 %	21.99 %

(2) *Sphingosine.* After the removal of lignoceric acid the alcoholic hydrolysate was diluted with water, kept on the water-bath for some time and the alcohol finally evaporated. Just as in the case of phrenosin, it was found that besides sphingosine a certain amount of methyl derivatives of sphingosine had been formed during the methyl alcoholysis.

(a) *Sphingosine sulphate* was separated from this mixture by its relative insolubility in cold alcohol, in which methylsphingosine sulphate is soluble. After repeated purification by conversion into the free base, sphingosine sulphate was obtained as a white hygroscopic powder, showing a melting point of about 245°, and decomposing with effervescence at 250°. For further identification the amino-nitrogen was estimated by van Slyke's method, using the micro-apparatus recently described.

Amino-nitrogen estimation. A solution of sphingosine sulphate in glacial acetic acid was employed, containing 0.1484 g. of the substance in 10 cc. solution. 2 cc. were used for each experiment and the nitrogen evolution was found to be complete in 25–30 minutes. After making the necessary

correction, 2.01 cc. nitrogen at 763 mm. and 15° C. were obtained from 2 cc. of the solution.

	Found	Calc. for (C ₁₇ H ₃₃ O ₂ NH ₂) ₂ H ₂ SO ₄
N	3.96 %	4.19 %

(b) The *easily soluble sulphate* was converted into the free base by the addition of potash to its alcoholic solution. After addition of water the base was extracted with ether and it crystallised at once on evaporation of the solvent. The free base was dissolved in absolute ether and gaseous hydrochloric acid was passed into the solution. It solidified to a mass of crystals of the hydrochloride which was filtered off and washed with ether and acetone. The substance was recrystallised from a mixture of alcohol and acetone; it crystallised in transparent plates, resembling cholesterol crystals.

(0.0700 g.; 0.1644 g. CO₂; 0.0707 g. H₂O.)

	Found	Calc. for mono- methylsphingosine hydrochloride	Calc. for dimethyl- sphingosine hydro- chloride
C	64.05 %	64.32 %	65.18 %
H	11.30 „	11.40 „	11.52 „

The substance melted sharply at 141°, whilst dimethylsphingosine hydrochloride melts at 132°. The low carbon percentage and the higher melting point suggest that the substance represents a monomethylsphingosine hydrochloride.

In this connection it may be mentioned that Thierfelder [1913] also obtained on hydrolysis of various kerasin fractions a hydrochloride which showed the abnormal melting point of 139°. As the material employed for hydrolysis still admittedly contained phrenosin, it is possible that the substance, which was considered as dimethylsphingosine, consisted of a mixture of this substance with monomethylsphingosine. This question, which is of importance for the elaboration of the constitutional formula of kerasin, requires further investigation.

(3) *d-Galactose*. The carbohydrate contained in kerasin has up till now not been definitely identified. Thudichum assumed it to be identical with cerebrose (galactose) which he had obtained from phrenosin. I obtained the carbohydrate in the crystallised condition and identified it with galactose by oxidation to mucic acid and by its characteristic methylphenylhydrazone.

The hydrolysate of 3.50 g. kerasin, from which the fatty acid and bases had been removed as above described, was made up to 250 cc. and examined

polarimetrically. Actual rotation measured in 2 dm. tube = $+0.48^\circ$. Whence galactose = 21.19 %. Calc. for 1 mol. galactose from $C_{47}H_{91}O_8N$ = 22.6 %. Considering the unavoidable losses in the lengthy operations of the estimation, the value found agrees well with the calculated figure.

From one-half of the solution the carbohydrate was obtained by removing the sulphuric acid by means of barium carbonate and concentrating the filtrate *in vacuo*.

The residue was taken up in methyl-alcohol and the sugar recrystallised from water, from which it separated in the forms characteristic of galactose. A small quantity of the crystals was oxidised with dilute nitric acid according to Tollens. The mucic acid obtained showed the melting point of 190° and was converted into the ammonium salt by means of ammonium carbonate. The dry ammonium salt gave on heating a strong pyrrole reaction.

To the other half of the final hydrolysate solid sodium acetate was added until the reaction ceased to be acid towards Congo-paper. A few drops of methylphenylhydrazine were then added and the hydrazone separated and recrystallised as described above under phrenosin. The hydrazone showed a sharp melting point of 191° .

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SUMMARY.

1. The galactoside phrenosin prepared from brain by the pyridine method is identical with Thudichum's phrenosin.
2. The substances described by various authors as pseudo-cerebrin, cerebrin, cerebron and *d*-cerebrin are identical with phrenosin.
3. Phrenosin is dextrorotatory and furnishes on hydrolysis: (1) phrenosinic acid ($C_{25}H_{50}O_3$), (2) sphingosine, and (3) galactose.
4. Kerasin is laevorotatory and furnishes on hydrolysis: (1) lignoceric acid ($C_{24}H_{48}O_2$), (2) sphingosine, and (3) galactose.
5. Constitutional formulae for the two galactosides of brain are suggested.

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