LVIII. THE GALACTOSIDES OF THE BRAIN. I.

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(Received Nov. 10th, 1913.)

Nearly forty years ago Thudichum [1874] recognised the existence in the brain of substances analogous in their constitution to the vegetable glucosides. He distinguished two principal representatives of this class which he called phrenosin and kerasin. Phrenosin being relatively easy to prepare in sufficient amount for further study, Thudichum succeeded in elucidating its constitution by an investigation of its hydrolytic cleavage products. The carbohydrate obtained on hydrolysis of phrenosin was originally called "cerebrose" by its discoverer, but was identified later with galactose [Thierfelder, 1890; Brown and Morris, 1890]. The group name "cerebroside," in analogy to "glucoside," was introduced by Thudichum on the assumption that cerebrose was the typical carbohydrate of the brain, but later on he himself adopted the name cerebro-galactoside. In view of the fact that the occurrence of these substances is not limited to the brain¹, the use of the general name "galactoside" seems to be preferable to the special term "cerebroside."

The work of subsequent investigators was limited, until quite recently, to the preparation by modified methods of the substances first obtained by Thudichum, without contributing to the knowledge of their cleavage products. Unfortunately a great confusion in the nomenclature of these substances has resulted from the failure of earlier workers to co-ordinate their results with each other and with those of Thudichum. It seems that too much importance was attached to minor differences in the figures of elementary analysis and to variations in the melting points of these substances of high molecular weight; it was mainly on this basis that new names like pseudo-cerebrin, cerebrin, homocerebrin, cerebrin were introduced for substances, which from the method of their preparation were nothing else than Thudichum's original phrenosin and kerasin, or mixtures of the two.

¹ Similar substances have since been obtained from pus (Kossel and Freitag), from blood (Bang and Forssmann), from adrenals (Rosenheim and Tebb), and also from certain mushrooms (Zellner), etc.

In the course of a prolonged investigation of these substances, carried out during the last six years, the author has been able to confirm Thudichum's main observations and has worked out a new method for the preparation of the galactosides from brain. The complete separation of the galactoside mixture into its two principal constituents, phrenosin and kerasin, was made possible for the first time by the help of a new physical test, depending on the behaviour of these substances under the polarising microscope, which will be described later.

The investigation of the optical activity of the two substances brought out an interesting difference between them. Whilst phrenosin, in confirmation of the previous observations of Thierfelder and Kitagawa [1906], proved to be dextro-rotatory, it was found that kerasin, when completely separated from all dextro-rotatory phrenosin, possessed a laevo-rotation. Levene and Jacobs [1912, 2] and subsequently Thierfelder [1913] have since described as inactive a "kerasin fraction" prepared by different methods and evidently not completely freed from phrenosin.

The two products were further subjected to hydrolysis and all the cleavage products were identified. As far as phrenosin is concerned, the results agreed with Thudichum's observations which had already been confirmed in the main outlines by Thierfelder's work [1904, 1905].

The complete hydrolysis of kerasin had hitherto not been carried out and its relationship to phrenosin had remained unexplained. In its elementary composition it agrees closely with phrenosin and yet it differs widely from it in appearance, solubility and other physical constants. Thudichum suggested that the essential difference between it and phrenosin might be due to a difference in the fatty acid radicle. Neither the fatty acid nor the carbohydrate nor the base had been isolated or identified by Thudichum. Levene and Jacobs [1912, 2] recently advanced the hypothesis that the two substances represented optical isomers.

My own results show that Thudichum's suggestion was correct and that kerasin contains a different fatty acid from phrenosin. On hydrolysis of kerasin, purified as far as possible, I obtained as the only fatty acid one of the composition $C_{24}H_{48}O_2$, which I was able to identify with lignoceric acid [1913]. The carbohydrate was found identical with *d*-galactose and the base with sphingosine, as was to be expected from Thudichum's preliminary work.

Phrenosin, on the other hand, gives rise to the optically active hydroxy-acid $C_{26}H_{50}O_3$, as well as to *d*-galactose and sphingosine¹.

¹ Thierfelder [1904] was the first to show that the fatty acid obtained by Thudichum from phrenosin represented a hydroxy-acid. Levene and Jacobs [1912, 1] satisfactorily explained the

In connection with this work the question was investigated in which form the galactosides occur in the brain, and it is proposed to deal with this fundamental question first, leaving the description of the methods used for the separation of phrenosin and kerasin, as well as for their purification and hydrolysis, for a subsequent communication.

I. THE GALACTOSIDES EXIST IN THE BRAIN IN THE FREE STATE.

Previous investigations leave the question undecided whether the galactosides occur preformed in the brain or whether they are split off from more complex substances during the process of preparation. Whilst some recent authors incline to the view that part at least exist preformed, others are of the opinion that, as only small quantities are obtainable directly from the brain without drastic methods, they owe their origin to the partial decomposition of a preformed complex substance. No experimental proof for either view has been brought forward.

It is evident that this question cannot be decided by the use of those methods hitherto employed for the preparation of the galactosides, in which either baryta is used for the removal of the phosphatides, or in which solvents like alcohol are used at their boiling temperature. In both cases a decomposition of a preformed combination is possible. It seemed feasible, however, to obtain conclusive evidence on this point by the use of an inert solvent which dissolves galactosides in the cold, and in which phosphatides and sulphonatides are insoluble. Such a solvent was found by Rosenheim and Tebb [1910] in pyridine.

In applying the pyridine method directly to brain, it is preferable previously to remove water and cholesterol by means of cold acetone [Rosenheim, 1906], and the unsaturated phosphatides, lecithin and kephalin, by means of ether or petroleum ether. As the same method was used subsequently for the isolation of the galactosides, the procedure adopted may be described here in detail.

differences in melting point of Thudichum's and Thierfelder's acid by showing that the acid existed in two isomeric modifications, of which the optically inactive one melts at 82-85° (Thudichum's "neurostearic" acid), whilst the dextro-rotatory form melts at 106-108° (Thierfelder's "cerebronic" acid). From a "kerasin fraction" Thierfelder [1913] has recently obtained an acid of the composition $C_{24}H_{48}O_2$; Levene [1913] has since identified a similar acid as lignoceric acid, which he isolated on hydrolysis of a "cerebrin fraction." Both Thierfelder's and Levene's fractions were optically inactive and contained therefore probably dextro-rotatory phrenosin admixed with the laevo-rotatory kerasin. Thierfelder, indeed, obtained 6% of cerebronic acid, the typical acid of phrenosin, on hydrolysis of his "kerasin fraction." The carbohydrate had not been identified by either of these workers.

10 kg. of finely minced ox brain were suspended in 10 litres of acetone and allowed to stand with frequent stirring for 24 hours at room temperature. The watery acetone extract was decanted and the brain pulp strained through several layers of fine muslin. At least six subsequent extractions with sufficient acetone to cover the tissue were made, until the last extract on evaporation yielded only an inappreciable amount of cholesterol. The total quantity of crude cholesterol obtained from these extracts varies between 240–260 g., i.e. about 2.5 % of the fresh brain.

The tissue was next spread in a thin layer on large glass plates, gently warmed from below, and freed from acetone by means of an air current (electric fan).

The dry, somewhat waxy powder was now subjected to extraction with cold petroleum ether. Five to six extractions were usually found sufficient to remove the unsaturated phosphatides, which may be obtained from the extracts by the usual methods. After the removal of the petroleum ether by an air current in the way described above, the tissue was passed through an Excelsior mill and was thus obtained as a fine cream-coloured powder, ready for the pyridine treatment. 10 kg. of fresh brain yield on the average 1300 g. (from 1200–1400 g.) of this powder, which seems to keep indefinitely in this condition.

For the preparation of the galactosides it was found convenient to work up 500 g. of this powder at a time. This quantity was covered with 1500 cc. of pyridine (B.P. 115°) and after having been warmed to 45° by being kept for about twenty minutes in a water-bath at 50°, it was rapidly cooled to room temperature. Although the galactosides are readily soluble in cold pyridine, the initial warming is necessary in order to allow the solvent to penetrate the tissue. Filtration proceeds easily by means of a large Buchner funnel. From their pyridine solution the galactosides were obtained by pouring it into 3–4 volumes of acetone¹. A bulky white precipitate is formed, from which the supernatant fluid can be easily decanted off after standing some time. The mother liquor deposits still further on cooling on ice. In the earlier experiments this deposit was filtered off separately, but as the amount was found to be very small, the pyridine-acetone mixture was cooled down directly to 0° in the later experiments.

The filtration and washing of the mixed galactosides under pressure proceeds very slowly and it is therefore advisable to filter through a plain filter. After thorough washing with acetone, the precipitate is suspended in acetone and only then filtered under pressure. After being dried in vacuo,

¹ The pyridine solution may, of course, be previously concentrated by distillation in vacuo.

the powder is extracted with ether in a Soxhlet, in order to remove the last traces of ether-soluble phosphatides.

The crude galactosides are thus obtained as a slightly yellowish powder, the average yield amounting after two extractions to 205 g. from 10 kg. of moist brain, i.e. 2 per cent.

It is of interest to compare this experimental yield with the theoretical. Unfortunately there is at present a lack of a reliable quantitative method for the estimation of galactosides and the data available in the literature as to the percentage of galactosides in normal brain are correspondingly scanty. Probably the method worked out recently by Lorrain Smith and Mair [1913], in which the galactosides are weighed as such, gives truer results than the older methods in which the reducing power of hydrolysed brain extracts served as a basis of calculation. Lorrain Smith and Mair state their results in percentages of the chloroform extract of dried brain. Taking the mean water content of brain as 78 %, I have calculated from their results the percentages of galactosides as 7.3 % in dry and 1.6 % in fresh normal human brain. This result agrees well with the experimental yield of 9.8 % in dry and 2 % in fresh brain, as stated above, if we consider that these figures refer to the crude product.

After having been recrystallised twice from 15 volumes of an alcohol-chloroform mixture (1:2) the substance was obtained as a white powder, which contained 1.68 % of nitrogen and only a very small percentage of phosphorus (0.08 %).

0.3000 g.; 2.36 cc. N/10 KOH by Neumann's method.
0.4893 g.; 5.86 cc. N/10 KOH by Kjeldahl's method.

The product represents, as will be shown in a subsequent communication, a mixture of at least two substances. For the purpose of the present investigation it was considered sufficient to show that it consists essentially of galactosides, and the substance was therefore subjected to hydrolysis without any further purification.

Method of estimation of the products of hydrolysis.

Hydrolysis was carried out in methyl alcohol solution with sulphuric acid, and the cleavage products estimated in a way similar to that described by Thierfelder [1905] for "cerebron." Galactose was estimated polarimetrically and the fatty acids (as esters) and bases (as sulphates) were collected as carefully as possible and weighed.

1 g. of the substance was dissolved in 50 cc. methyl alcohol containing

5 cc. concentrated sulphuric acid and boiled under a reflux condenser on a water bath for six hours. After standing overnight, white glittering scales of the esters (and free fatty acids) had crystallised out. The flask was kept in the ice-chest for some hours and the crystals were filtered off, washed with cold methyl alcohol, dried in vacuo and weighed. To the filtrate water was added, and the clear solution boiled for some time in order to hydrolyse the methyl galactoside formed during hydrolysis. The alcohol was evaporated on the water bath. During the concentration of the watery solution, oily droplets of the sulphates of the bases appeared, which solidified on cooling. The deposit was filtered, taken up in boiling alcohol and the solution evaporated in vacuo. The residue consisting of the sulphates of sphingosine (and dimethylsphingosine) was weighed after having been dried to constant weight in vacuo. The final filtrate was made up to 100 cc. and examined polarimetrically.

Previous experiments had shown us that the results of galactose estimations by the polarimeter agreed with those made by Kjeldahl's gravimetric method.

Experiment I. 1 g. substance gave 0.303 g. esters and 0.620 g. sulphates of bases. Galactose solution: actual rotation measured = $+0.33^\circ$ (mean of six readings) in 2 dm. tube. Whence galactose = 20.39% [see Landolt, 1892, p. 452].

Experiment II. 1 g. substance of a different preparation gave 0.280 g. esters. Galactose solution: actual rotation measured = $+0.34^\circ$ in 2 dm. tube. Whence galactose = 21.01%.

d-Galactose was identified in the final solution by means of methylphenylhydrazine. The free mineral acid was neutralised with solid sodium acetate and methylphenylhydrazine was added. After standing at room temperature for some hours, crystals of the hydrazone settled down. They were filtered off and recrystallised from alcohol. The white crystals melted sharply at 191° . The melting point of galactose methylphenylhydrazone is given by Neuberg [1907] as 191° [see also Fränkel, 1910]. From another portion of the hydrolysate, galactose was prepared as such by the usual methods and identified as mucic acid by oxidation with nitric acid. The dry ammonium salt of the mucic acid thus prepared gave on heating a strong pyrrole reaction.

The results are given in the following table (page 610), which shows also for comparison the figures obtained by a similar method by Thierfelder from the hydrolysis of more or less purified galactosides.

It will be seen from these results that the cleavage products obtained on hydrolysis of the galactoside mixture show a close resemblance in their nature, as well as in their quantitative distribution, with those obtainable from the

	Galactoside mixture prepared by the pyridine method		Phrenosin ¹ (cerebron)	Kerasin fraction
	I	II		
Nitrogen %	1.68	—	1.76	1.61
Galactose %	20.39	21.01	19.88	19.35
Esters + fatty acid %	30.3	28.0	38.7	29.6
Bases (as sulphates)	62.0	—	50.3	57.2

¹ The figures given represent the mean values calculated by me from Thierfelder's figures.

galactosides isolated by a different method. This result therefore seems to justify the conclusion that the product obtainable from brain by extraction with cold pyridine, consists practically entirely of galactosides. In their preparation any possible decomposition of a preformed complex substance has been carefully avoided by limiting the time of extraction to a minimum and by employing an inert solvent at a low temperature. As, further, the amount obtainable experimentally agrees very well with the theoretical yield, we must assume that the whole of the galactosides exist in the brain in the preformed state.

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

SUMMARY.

- (1) A new method for the preparation of galactosides from brain by means of pyridine is described.
- (2) Evidence is brought forward to show that the galactosides exist in the brain entirely in the preformed condition.

REFERENCES.

- Brown, Horace T. and Morris, G. H. (1890), *J. Chem. Soc.* **57**, 57.
 Fränkel, S. (1910), *Biochem. Zeitsch.* **26**, 41.
 Landolt, H. (1892), *Das Optische Drehungsvermögen*, Braunschweig.
 Levene, P. A. and Jacobs, W. A. (1912, 1), *J. Biol. Chem.* **12**, 381.
 — (1912, 2), *J. Biol. Chem.* **12**, 389.
 — (1913), *J. Biol. Chem.* **15**, 359.
 Neuberg, C. (1907), *Biochem. Zeitsch.* **3**, 531.
 Rosenheim, O. and Tebb, M. C. (1910), *J. Physiol.* **40**, Proceedings, i.
 — (1906), *J. Physiol.* **34**, 104.
 — (1913), *Trans. Internat. Congress of Med. Sect. II*, 626.
 Smith, J. Lorrain and Mair, W. (1913), *J. Path. Bact.* **17**, 609.
 Thierfelder, H. (1890), *Zeitsch. physiol. Chem.* **14**, 209.
 — (1904), *Zeitsch. physiol. Chem.* **43**, 21.
 — (1905), *Zeitsch. physiol. Chem.* **44**, 366.
 — and Kitagawa, F. (1906), *Zeitsch. physiol. Chem.* **49**, 288.
 — (1913), *Zeitsch. physiol. Chem.* **85**, 35.
 Thudichum, L. J. W. (1874), *Reports of the Med. Off. of the Privy Council and Local Govt. Board*, New Series, No. III.
 — (1882), *J. pr. Chem.* **25**, 19.
 — (1901), *Chem. Konstitution d. Gehirns d. Mensch. u. d. Tiere*, Tübingen.