XV. THE GALACTOSIDES OF THE BRAIN. II. THE PREPARATION OF PHRENOSIN AND KERASIN BY THE PYRIDINE METHOD.

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The methods hitherto used for the preparation of the galactosides from brain may be divided into two groups. In one group of methods, which may be traced back to Couerbe's work, a separation of the galactosides from the phosphatides is effected by purely physical means, mainly by the help of such solvents as alcohol (Thudichum), glacial acetic acid (Koch), methyl alcohol-chloroform (Wörner and Thierfelder), etc. In the second group of methods the relative resistance of the galactosides to hydrolysis by baryta is used to separate them from the easily saponifiable phosphatides. The latter method which dates back to the work of Müller has been applied directly to the brain (Parkus) or to the mixture of galactosides and phosphatides etc., which is obtained by cooling an alcoholic extract of brain (Kossel and Freytag, Thierfelder, Fränkel, Lapworth).

The product obtained by both methods consists of a mixture of at least two galactosides, a fact which was first recognised by Thudichum. To the latter investigator belongs also the credit of having introduced the only available method for their separation, which may be termed a temperature fractionation method.

Both groups of methods possess one inherent disadvantage, which has considerably retarded the study of brain lipoids in general. Whilst allowing the separation of the galactosides, they make it most difficult or even impossible to isolate the saturated phosphatides which are present in brain. Indeed since Thudichum, all the subsequent investigators, until quite recently, limited their work to the preparation of the galactosides, neglecting altogether the phosphatide moiety of the original product¹.

¹ Thudichum isolated one of these phosphatides, called by him sphingomyelin, by means of its cadmium salt, and it has since been prepared by other methods by Rosenheim and Tebb. According to unpublished investigations of Rosenheim and Tebb, the cadmium method is altogether unsatisfactory.

Further, none of the authors who used the baryta method seem to have taken into consideration the fact that the galactosides are more or less hydrolysed by prolonged boiling with baryta in aqueous or alcoholic solution. Only quite recently it has been shown by Thierfelder and Loening [1912] that relatively short boiling with aqueous baryta does not split off galactose from these substances. It remains, however, a significant fact that only those observers who used the alcoholic baryta method found a high nitrogen percentage in their products. This fact suggests that their preparations contained certain amounts of the nitrogenous base sphingosine, set free by partial hydrolysis from the galactosides or from the phosphatide sphingomyelin. This possibility must be borne in mind, especially as the alcoholic baryta method has recently again come to the fore [Fränkel, 1911; Lapworth, 1913], without apparently any previous investigation of the action of alcoholic baryta on the native galactosides.

Principle of new method.

The difficulties and failures of the older methods used for the study of the brain lipoids may be traced back to the general use of alcohol as the primary solvent. The solubilities in alcohol of the various substances composing the complex lipid mixture, as it occurs in brain, are such that their separation has baffled investigators ever since Vauquelin first applied this solvent to brain over 100 years ago. The problem becomes much simplified by discarding altogether the use of alcohol as a primary solvent, and by applying to the brain the principle of selective extraction as first suggested by Rosenheim [1906], and subsequently worked out by Rosenheim and Tebb [1909, 1910]. The method consists shortly in extracting brain, suitably dehydrated, at various temperatures with solvents which as far as possible dissolve out only one group of lipoids at a time. The method has so far led to the separation of the following lipoids:

- (1) Cholesterol (by extraction with cold acetone¹).
- (2) Lecithin and kephalin (by extraction with ether or petrol ether).
- (3) Galactosides (by extraction with cold pyridine).
- (4) Sphingomyelin and sulphatide (by extraction with warm pyridine).

The method has already been described in detail in a previous communication in this journal [1913], as far as the preparation of the crude galactosides is concerned.

¹ Acetone has subsequently also been adopted by S. Fränkel as the primary solvent in his method for the preparation of brain lipoids. After the removal of the unsaturated phosphatides, lecithin and kephalin, this investigator again reverts to the use of alcohol and proposes to separate the mixture of galactosides, saturated phosphatides and sulphatides by the use of the old methyl alcoholic baryta method [1911].

Nomenclature.

In his "Proposals for the nomenclature of the lipoids" the author has already stated in this journal [1909] the reasons for discarding the names cerebrin, pseudo-cerebrin, cerebron and homo-cerebrin. It was proposed, following the suggestion of Posner and Gies [1906], to retain the original names phrenosin and kerasin, introduced by Thudichum, for the two principal galactosides isolated by him from brain.

This nomenclature is now generally accepted by most writers on the subject, and it seems only logical to extend it to the cleavage products of these substances. The author has therefore followed Posner and Gies [1906] in adopting the name "phrenosinic acid" for the fatty acid obtained on hydrolysis of phrenosin. The term "kerasinic acid" proposed by Thierfelder for the corresponding acid from kerasin seems however to be unnecessary since this acid has been identified by Rosenheim and by Levene with the longer known natural lignoceric acid.

The separation of phrenosin and kerasin.

The difficulty of separating the galactoside mixture into its constituents is probably due to several causes, the most important of which are the following: (1) the close resemblance in their chemical constitution (see later); (2) the close resemblance of their physical properties, such as solubility etc.; (3) their chemical inertness, which prevents the formation of any characteristic derivative suitable for their purification¹; (4) the remarkable property of these lipoids to exist in the liquid crystalline condition (see next communication) must also be held responsible to a large degree for the difficulties of their separation, especially since O. Lehmann, the discoverer of this interesting state of matter, has clearly demonstrated the easy miscibility of liquid crystals; (5) the absence of any criterion by means of which the completeness of the separation can be judged. Melting point estimation and elementary analysis are practically useless and may even be misleading in this case as has been clearly demonstrated by the experience of Thierfelder and of Levene and Jacobs².

¹ I have, however, found that these galactosides, when treated in pyridine solution with benzoyl chloride, easily give benzoyl products. A method for the separation of phrenosin and kerasin might be based on the different solubilities of their benzoyl derivatives.

² In spite of the close agreement of many analyses of "cerebrin," Thierfelder and Loening were able to show later [1910] that it contained an admixture of another galactoside. Levene and Jacobs [1912] were so much struck by the agreement of their analyses of "cerebrine" and kerasin, that they considered them for this, amongst other reasons, to be stereo-isomeric substances.

The following observations, made by Rosenheim and Tebb six years ago in connection with some other work on brain lipoids [1908], are therefore of considerable value for the separation of phrenosin and kerasin.

(1) Phrenosin (in pyridine solution) was found to be dextrorotatory, whilst kerasin is laevorotatory in the same solvent.

(2) The behaviour of the two substances under the polarising microscope is characteristically different. It allows the detection of phrenosin in presence of kerasin, and *vice versa*, by a simple test which will be described later as the selenite-plate test.

By the help of these two tests, the progress of the separation of the galactoside mixture may be controlled. For the actual separation the principle of temperature fractionation, introduced by Thudichum, was made use of. As, however, the previous experience of Rosenheim and Tebb [1908] had shown that by the use of alcohol, which was the only solvent employed by Thudichum, a complete removal of the phosphatides is practically impossible, I have successfully used acetone containing 10% of water for the primary fractionation.

The mixture is thus separated into: (a) *the phrenosin fraction*, deposited on cooling the acetone solution, from 50° to 36°, and (b) *the kerasin fraction*, deposited from the clear decanted mother liquor of (a) on cooling from 28° to about 0°.

It can easily be demonstrated by the use of the selenite-plate test that the phrenosin fraction still contains kerasin, and that phrenosin is present in the kerasin fraction. Their complete separation cannot be effected by the repetition of the temperature fractionation process with any single solvent. A series of fractionation experiments was therefore carried out with various mixtures of solvents and the products tested by the selenite-plate test as well as by the polarimeter. The following procedure was finally adopted.

Phrenosin is obtained free from kerasin by recrystallisation of the phrenosin fraction from a glacial acetic acid-chloroform mixture (3:2) at 37°. After the third recrystallisation from this solvent, it is usually found that the filtrate from the deposit formed at 37° no longer deposits kerasin when cooled to room temperature. The last traces of phosphorus are also removed by this solvent.

The selenite-plate test, however, still reveals at this stage the presence of small quantities of kerasin, which are removed by repeated temperature fractionation from a mixture of acetone-chloroform (3:2). The last filtrate from the deposit at 37° must remain perfectly clear on cooling to room temperature.

The product is finally once more recrystallised from acetone containing 10 per cent. pyridine.

Kerasin is obtained free from phrenosin in a similar way from the kerasin fraction by using the same solvents. In this case, however, the superficial criterion of the removal of phrenosin is given by the fact that the solution remains perfectly clear at 37° and only begins to deposit when cooled to 28°. The final product, recrystallised again from acetone-pyridine, should be laevorotatory, and when examined under the polarising microscope, show the complete absence of phrenosin.

A comparison of the substances thus obtained with phrenosin and kerasin prepared previously by Rosenheim and Tebb [1908] according to Thudichum's original method, showed a close agreement in elementary composition, melting point, optical activity, appearance under the polarising microscope and in their hydrolytic cleavage products. As far as our present knowledge admits, the identity of phrenosin and kerasin prepared by Thudichum's method and by the new pyridine method may be considered as proved.

EXPERIMENTAL.

The crude galactoside mixture, the preparation of which has already been described in detail in Part I of this series of communications [1913], was used for the isolation of the substances to be described. After two recrystallisations from an alcohol-chloroform mixture, it still contained a small percentage (0·08 %) of phosphorus. As the selenite-plate test was found to be most useful for controlling the progress of the separation of this mixture it may be described next.

The selenite-plate test. Both phrenosin and kerasin separate from a warm 10 % solution in pyridine on gradual cooling in the form of spherocrystals, possessing approximately the same refractive index as the solvent. They are therefore scarcely visible under the microscope in ordinary light. In polarised light, with crossed nicols, they stand out bright on the black background and show well-defined crosses. If a selenite-plate (Red I) is placed below the stage and immediately above the polariser in such a way that its axis lies diagonally to the planes of polarisation of the crossed nicols, a characteristic difference between phrenosin and kerasin will be noticed at once. On the red background the spherocrystals appear to be divided into quadrants, of which two opposite ones show the addition colour, blue, whilst the two others show the subtraction colour, yellow. But, while the spherocrystals of phrenosin, under the above conditions, show the blue colour in

Fig. 1
Spherocrystal of phrenosin

Fig. 2
Spherocrystal of kersin

Fig. 3
Spherocrystal of a phrenosin-
kersin mixture

Reproduced from colour photographs (Lumière's process), taken in polarised light with a Selenite plate (Red I). The arrow indicates the direction of the axis of the Selenite plate.

the upper right and lower left quadrants (Fig. 1), the reverse is the case when the spherocrystals of kersin are observed under exactly the same conditions (Fig. 2). Or, in other words, to use the nomenclature proposed by Göthlin [1913], the paragonal quadrants of the phrenosin spherocrystals show the addition colour, and the epigonal quadrants the subtraction colour, whilst the kersin spherocrystals show the reverse behaviour. If we accept the current views according to which a spherocrystal is composed of radially arranged prisms, we may say that a spherocrystal of phrenosin is positively anisotropic, i.e. it is composed of optically positive prisms which are radially arranged. Kersin on the other hand yields negatively anisotropic spherocrystals under the same conditions.

By means of this simple physical test, it is possible to establish in a very small sample the complete removal of kersin from a phrenosin preparation, and *vice versa*.

It was found that from a galactoside mixture (i.e. either from any impure phrenosin or kersin fraction, or from an artificial mixture), spherocrystals of both kinds are formed. They usually appear at first as separate crystals, varying in size from 0.05 to 0.5 mm. If the preparation is left for some hours, however, a kersin crystal may deposit round a spherocrystal of phrenosin, or *vice versa*. A typical crystal of this kind, obtained from a phrenosin fraction, is reproduced from a colour photograph in Fig. 3. The limit of the sensitiveness of this test has not yet been established quantitatively. That it is, however, sufficiently sensitive for practical purposes, follows from the fact that a laevorotatory kersin fraction, which was found to be free from phrenosin by the selenite-plate test, was also found on hydrolysis to be free from phrenosinic acid, the typical fatty acid of phrenosin.

In order to economise the valuable material, the test was carried out by dissolving a very small quantity (8–10 mg.) of the substance in a small test tube (0.5 × 4 cm.) by adding two drops of pyridine and warming to about 37°. A drop of the solution was transferred by means of a warm capillary pipette to a warmed slide, covered and allowed to cool gradually. A layer of spherocrystals forms at first round the edges of the coverslip, thus preventing further evaporation of the solvent, a fact which makes it possible to preserve the preparation for reference.

Fractionation of the galactoside mixture from acetone.

Previous work of Rosenheim and Tebb [1907] in connection with brain lipoids had demonstrated the advantage of acetone as a solvent for galactosides¹. I have since found by a series of systematic experiments (omitted to save space), that the solvent power of acetone for galactosides is greatly increased if it contains 10 % of water.

The finely powdered galactoside mixture was therefore treated in portions of 50 g. with 3500 cc. of 90 % acetone in a water-bath kept at 56°. Only one extract was made and the insoluble part (about 15 %) has not yet been further examined. The clear acetone solution was allowed to cool in an incubator at 37°. After 16–20 hours a crystalline deposit, partially adhering to the sides of the glass vessel (phrenosin fraction), had formed, from which the supernatant fluid was easily decanted through a filter warmed to 37°. The filtrate was allowed to stand for 24 hours or longer in an ice chest, and the bulky gelatinous precipitate considered as the kerasin fraction.

The precipitates were filtered under pressure, washed with acetone and dried *in vacuo*. The kerasin fraction amounted to about 50 % of the phrenosin fraction, but the actual amount of kerasin contained in it is naturally much smaller, as only a rough separation is effected by this treatment.

Phrenosin.

32.5 g. of the phrenosin fraction were recrystallised from ten volumes of glacial acetic acid-chloroform (3:2). The finely powdered substance was first dissolved in 120 cc. of chloroform at about 60°, and to the solution was added 180 cc. of acetic acid previously warmed to the same temperature.

The clear solution was kept in an incubator at 37° over night, the deposit filtered and washed at 37° with the acetic acid-chloroform mixture. The moist precipitate was again dissolved in 200 cc. of the solvent and the solution treated in the same way. (The mother liquors of these two recrystallisations deposited on cooling to room temperature a considerable amount (6.64 g.) of a gelatinous precipitate, which was worked up with the kerasin fraction.)

The product deposited at 37° was granular and easy to filter. When recrystallised a third time in the same way, the mother liquor no longer

¹ This solvent has since been used for the same purpose by L. Smith and Mair [1910], and by Thierfelder and Loening [1912].

deposited on cooling to room temperature. After drying *in vacuo* the product weighed 20.1 g.

The selenite-plate test at this stage reveals only a small quantity of kersin. A phosphorus estimation (Neumann's method) was intended, but 0.5532 g. of the substance carried through the process gave no trace of precipitate with the molybdic acid reagent, and the substance was therefore to be considered as phosphorus-free.

As the acetic acid-chloroform mixture did not seem to effect any further separation of kersin, the product was now recrystallised from 100 volumes of acetone-chloroform (3:2). 19 g. of the product were first dissolved in 760 cc. chloroform and 1140 cc. warm acetone were added. The granular deposit, filtered at 37°, was twice more recrystallised in the same way. It then appeared to be perfectly uniform, as tested by the selenite plate.

In order to test its homogeneity, however, the product was separated into two fractions by using acetone-pyridine (equal parts) as a solvent. 13 g. were dissolved at 45° in 130 cc. pyridine and 130 cc. warm acetone were added. After keeping the solution for two hours at 35°, the deposit formed at this temperature was filtered off, and the solution cooled to room temperature. A further precipitate formed. The two fractions will be designated as phrenosin Ia (6.5 g.) and phrenosin Ib (5.2 g.).

From 30 g. phrenosin fraction of a different preparation there were obtained: 5.3 g. phrenosin II a and 4.7 g. phrenosin II b.

All the preparations, before being subjected to detailed investigation, were finally recrystallised from a large volume of acetone containing 10 per cent. of water.

All the preparations gave identical results on elementary analysis. They showed the same behaviour on melting and possessed the same optical activity. Further they gave on hydrolysis qualitatively and quantitatively the same products. These facts furnish strong evidence that a uniform substance had been isolated.

The product was further compared with phrenosin prepared according to Thudichum's original method. Thudichum fractionated his "white matter" from absolute alcohol at 37° and at room temperature. The fraction obtained at 37° serves for the preparation of phrenosin. Before or after the fractionation process, the product is treated with an ammoniacal alcoholic lead acetate solution in order to remove substances of the "cerebrin acid" type, which form insoluble lead salts. Thudichum's directions were closely followed, but as he had experienced considerable difficulty in obtaining his substance completely free from phosphorus, it was found advisable, in the light of later

experience, to use other solvents (glacial acetic acid, chloroform) as well as alcohol after the primary fractionation process. The lead treatment was carried out at the end of the fractionation process. Only traces of the "cerebrin acids" were found to be present. 90 g. "white matter," free from cholesterol, yielded finally 10 g. phrenosin. The product was free from phosphorus and sulphur and agreed in every detail with phrenosin prepared by the new pyridine method.

Kerasin.

From the kerasin fraction (see above), kerasin was separated in the following way: portions of 10 g. were dissolved in 40 cc. chloroform at 50° and to the solution were added 60 cc. glacial acetic acid previously warmed to about 60°. The solution remained clear until the temperature had fallen to 40° and was then kept in an incubator at 37°. A granular white layer, mainly phrenosin, collected on the surface and was filtered off at 37°. The filtrate began to deposit on cooling to 26° and solidified finally to a gelatinous mass. After filtering without pressure, and washing with the chloroform acetic acid mixture, the product was suspended in acetone and could be filtered under pressure. The product, when dry, weighed only 5 g. and was again recrystallised from 50 cc. of the chloroform acetic acid mixture as above described. Only 0.5 g. came down at 37°, the main quantity depositing at room temperature. The process was repeated twice more, when it was found that the solution no longer deposited at 37°, even when kept at that temperature for many hours. At this stage the selenite-plate test showed that the substance consisted practically only of kerasin, only a few isolated spherocrystals of phrenosin appearing after some hours.

Further purification was obtained by continuing the recrystallisation from 20 volumes of an acetone-pyridine mixture (equal parts). Three grams were dissolved in 30 cc. pyridine and 30 cc. acetone warmed to 45° were added. Only a faint cloud appeared on cooling to 37° in the incubator. This small deposit of phrenosin weighed 0.01 g. when filtered and dried *in vacuo*. The main filtrate began to deposit at 28° and was allowed to cool to room temperature before filtration. This process was repeated and the product finally recrystallised from a large volume of 90% acetone containing 2% pyridine.

The kerasin preparations were considered pure when only kerasin spherocrystals were found by the selenite-plate test and when their optical activity was not less than $[\alpha]_D = -2^\circ$.

The separation by these purely physical means necessarily entails large losses, but leads finally to a pure product, a fact which was confirmed by the investigation of the hydrolytic cleavage products. From 10 g. of the original kersin fraction, there were obtained on the average 1.36 g. kersin (from 10–15 %).

The physical appearance of kersin when depositing from its solutions is characteristically different from that of phrenosin. Whilst the latter usually deposits in loose granular masses, kersin forms a coherent jelly which contracts on shaking. When filtered and dried *in vacuo*, it can easily be powdered, but when allowed to dry in air it forms a translucent white wax-like substance, thus fully justifying the name given to it by Thudichum.

Kersin was also prepared according to Thudichum's method from the kersin fraction of "white matter" (see above). It was subjected to the lead and cadmium treatment as described by Thudichum. It was found that only minute traces of substances precipitable by these reagents were present, no doubt owing to the fact that mixtures of solvents (see above) were used for recrystallisation instead of alcohol alone. The observation of Thudichum with regard to the change of solubility in alcohol as purification proceeds was fully confirmed. A product which contains only traces of phrenosin begins to deposit kersin even at 37°, while originally, in presence of a large proportion of phrenosin, it only deposits below 28°.

From 90 g. cholesterol-free "white matter," there were obtained finally 5 g. kersin.

The product was free from phosphorus and sulphur and agreed in its composition and all its properties with kersin prepared by the pyridine method.

The results of the elementary analysis, the optical activity and hydrolytic cleavage products of phrenosin and kersin will be described in a subsequent publication.

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SUMMARY. .

1. The galactoside mixture obtained by extraction of brain with cold pyridine can be separated into a phrenosin and a kersin fraction by temperature fractionation from acetone.
2. Phrenosin is obtained by recrystallisation of the phrenosin fraction

at 37° from chloroform-glacial acetic acid mixture, followed by recrystallisation from a chloroform-acetone mixture.

3. Kerasin is obtained from the kerasin fraction by recrystallisation at room temperature from chloroform-glacial acetic acid mixture, followed by a pyridine-acetone mixture.

4. A test (the selenite-plate test) is described, by means of which the progress of purification is controlled.

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