BRAIN LIPINS OF NORMAL RATS

by

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INTRODUCTION

The study of nervous tissue is of interest chiefly because it is this tissue which controls and coordinates to a large extent the various parts of the animal organism, with its many sorts of tissues, and because it has the property of informing the organism as to the nature of its environment. Along with these properties, there occurs in the higher animals the property of consciousness, memory, and thought. The direction of evolution, though it diverges in many directions in most particulars, has been in almost a straight line toward a larger and more complex nervous system which would enable the animal to confront and modify its environment rather than to be shaped by the blind forces which sweep across the world.

The high development of a mammal's brain may be considered, then, to be the climax of the history of life.

Considerations of this sort have led Mathews to entitle the chapter of his "Physiological Chemistry" which deals with this psychic material "The Master Tissue of the Body" and Walter to designate the chapter of his "Biology of the Vertebrates" which describes the nervous tissue as "The Dominating Factor."

That there is a relation of this important substance to

the intelligence of the animal is obvious. Accordingly, we would suppose the psychologists and the physiologists to have been diligent in their study of the nature of nervous tissue. We would think that dieticians would be eager to study the relations between nutrition and the development of the brain. We would expect physiological chemists to have done much research on this pace-setter for the other metabolic activities.

Apparently, however, such work as has been done has failed to produce comprehensive definite results. The neurologists, anatomists, and physiologists have often been technically unequipped to undertake the study. The nutrition workers have been busy at other problems, and chemists have had no enthusiasm for working with such complex substances which could not be obtained in good crystalline condition or isolated as single, pure compounds. The most sustained effort to link the psychic state of the animal's nervous system with the chemical constitution of the tissue is in the study of narcosis. Even here the results obtained have been so conflicting that few definite conclusions are possible.

Further investigation of the constituents of the brain substance is therefore justified. Any advance on the knowledge of the chemistry of nervous tissue will make more

available to the biological workers methods of correlating their observations.

THE CHEMICAL COMPOSITION OF NERVOUS TISSUE

The nervous system of the body appears to be made up of specialized cells called neurones, which are the true stimuli-conveying units of the organism, and of the supporting neuroglia cells and of the non-cellular sheaths of the nerve fibers. As the brain and spinal cord present conveniently isolated nervous tissue, they are used as raw material in analysis.

It might be supposed that nervous tissue contains some rare element which would endow it with its unusual properties, but examination shows that it contains the same elements as the other body tissues. The adult human brain contains about 78.9 per cent water. In general, nervous tissue contains a somewhat higher proportion of water than the other tissues. The proteins of the brain are not noticeably different from the other body proteins. They do not occur in as great amounts as elsewhere, composing about 37.1 per cent of adult human brain when calculated on the dry matter. The proteins probably make up those parts of the brain which serve as framework on which the constituents which give the brain its psychic properties are arranged. The extractives

are a group of inorganic and organic water-soluble compounds which occur also in other sorts of tissues. Presumably they exist normally in the brain, although there is some question as to whether they exist in the brain as the separate individuals isolated upon analysis. No special function has been found for them. The extractives contain such compounds as hypoxanthine, caprine, lactic acid, formic acid, succinic acid, inosite, and the phosphate, chloride, and sulfate salts of calcium, potassium, zinc, iron, copper, magnesium, and sodium.

The factor which differentiates nervous tissue from other sorts of tissue is the high proportion of alcohol- and ether-soluble substances known as lipins or lipoids. A lipin may be defined as a material soluble in the common organic solvents, insoluble in water, and related in some way to the normal functioning of an animal cell. Although these substances are insoluble in water and contain fatty acids and glycerol, they are not neutral fats. In addition to these components of neutral fats, they contain certain peculiar nitrogenous bases, and may contain combined phosphoric acid, galactose, and combined sulfuric acid. It is probably these fat-like materials of the brain which give it its special qualities, for the lipins occur only to a small extent elsewhere but make up from 30 to 35 per cent of the adult human brain, calculated on the dry basis.

The lipins are difficult to isolate as pure substances. It has even been said that no absolutely pure brain lipin compound has ever been prepared, with the exception of the sterols (73) (80). The large number of so-called compounds listed in the literature together with the conflicting claims of various workers lead one to give considerable credence to this statement.

The brain lipins may be divided into three main groups, the phosphatides, the cerebrosides, and the sterols. In addition, there is a group of sulfur-containing compounds whose exact characters are uncertain.

The phosphatides derive their name from the fact that they all contain combined phosphoric acid. They may be divided into several groups of compounds. The structures and constitutions of the individuals of any single group are similar. Perhaps the most familiar group is the lecithins. Although this group is usually referred to as "lecithin", meaning a single compound, it probably is a mixture of a number of different individuals. Upon hydrolysis, a lecithin yields glycerylphosphoric acid, two fatty acids, and a member of the betaine group, choline. The structure of choline has been ascertained by synthesis; it is trimethyl, hydroxyethyl, ammonium hydroxide. The fatty acids obtained upon hydrolysis are the saturated palmitic and stearic acids, and the unsaturated oleic and

arachidonic acids. According to Mathews (83), Thudichum (107) states that the nitrogenous base of the lecithins is not choline, but neurine. According to Levene and Rolf (74), the usual formula given for the lecithins is that first suggested by Strecker (105). It shows the two fatty acids joined in an ester structure to two carbon atoms of the glycerol, and phosphoric acid combined with the third carbon atom in an ester structure. The hydroxy group of the ethyl radical in choline is combined with an acid radicle of phosphoric acid in an ester structure, to complete the molecule. The number of possible lecithins is ten, limiting all possibilities to the fatty acids mentioned and the same arrangement of ester structure. Of course, other fatty acids than those identified may sometimes be present; the number of lecithins would be increased accordingly. There is the possibility that the middle carbon atom of the glycerol radicle is asymmetric, which would allow d- and l-forms of each of the lecithins. This aspect of lecithin structure is discussed by Levene and Rolf (74).

The cephalins are a group of phosphatides whose constitution is similar to that of the lecithins. The fatty acids obtained upon hydrolysis of "cephalin" are stearic, oleic, cephalinic, linolic, linolenic, carnaubic, and arachidonic acids. The nitrogenous base usually assigned

to it is kolamine, amino ethyl alcohol. Other bases, such as β-hydroxy, α-amino butyric acid may be present, according to MacArthur (75). The lecithins and cephalins are usually classified as "unsaturated phosphatides." According to Backlin (3), this nomenclature is due to Fränkel.

Paramyelin is the name given to a third phosphatide, or group of phosphatides. According to Mathews (83), it consists of neurine, glycerolphosphoric acid, and two unidentified acids. Myelin is a little-known phosphatide, having properties similar to those of paramyelin. Its hydrolysis products are unknown.

Amidomyelin is a phosphatide of uncertain composition. It has two nitrogen atoms to one phosphorus atom, and so is called a di-amino mono-phosphatide. Its hydrolysis products are unknown, also.

Sphingomyelin is a di-amino mono-phosphatide of well-characterized chemical individuality. Upon hydrolysis it yields phosphoric acid; two nitrogenous bases, choline and sphingosine; and two fatty acids, lignoceric acid and a hydroxy-acid, possibly a hydroxy-stearic acid according to Mathews (83). The structure of sphingosin has received study by Klenk (50) (49); his formula for sphingosin is $CH_3(CH_2)_{12}.CH{:}CH.CH(NH_2).CH(OH).CH_2(OH)$. According to Mathews (83), the structure of sphingomyelin has been suggested by Levene as being an ester of phosphoric acid with

choline and sphingosine, with the fatty acid joined to the amine group of sphyngosine in a peptid linkage.

Assurin is a di-amino di-phosphatide of uncertain constitution. It seems to have a phosphorus free amino-lipotide united with it. The sphingomyelins are grouped by Backlin (3) under the heading "saturated phosphatides." They make up a large proportion of brain lipins.

The cerebrosides, or glycolipids, are all alike in possessing a unique component--a carbohydrate which appears to be galactose. Rosenheim has identified galactose as a constituent of the cerebrosides by forming its methyl-phenyl-hydrazone derivative (98). The presence of other sugars in the cerebrosides has not been investigated. The cerebrosides consist of a number of compounds, such as phrenosin, kerasin, nervon, cerebrin, cerebron, protagon, etc. It is quite likely that these "compounds" are simply mixtures of chemical individuals. Levene and Jacobs (73) make the following statement in regard to this question: "Since the three cerebrosides are distinguished principally by difference in solubility and since there is no evidence to the effect that they are composed of different elements, one may feel justified in advancing the hypothesis that the three cerebrosides are all mixtures of stereoisomeric substances. The difference in the solubility of the various substances may be determined by the proportion of the

optically active substances. It would perhaps be simpler to abandon most of the nomenclature and to refer to cerebrosides as d-cerebrin, l-cerebrin, and d-l-cerebrin. Of course, it is still necessary to discover a method by which a separation could be accomplished conveniently. Cerebrine, cerebron, and phrenosine correspond to d-cerebrin; kerasine and homocerebrin to d-l-cerebrin. Of course the possibility is not excluded that the lipoids of the brain contain cerebrosides of an entirely different order." Levene and Jacobs (72) have prepared the isomers of cerebronic acid and have discussed their properties. According to Klenk (48), cerebronic acid is a twenty-four carbon acid.

When a cerebroside is hydrolyzed, a nitrogenous base, sphingosine; a carbohydrate, galactose; and a fatty acid are obtained. The fatty acid may be lignoceric, cerebronic, phrenosinic, nervonic, or an unidentified twenty-four carbon acid. This last acid has been called "Klenk's acid." It is not improbable that some of the acids mentioned are identical with each other. The investigation of Klenk and Härle (51) indicates that the galactose and sphingosine are joined together in the same way in all the cerebrosides. Klenk proposes to call this grouping "psychosin." The cerebrosides contain no phosphorus.

Phrenosin is one of the two best known cerebrosides. It is made up of psychosin and a saturated fatty acid,

phrenosinic acid, whose formula is believed to be $C_{25}H_{50}O_3$. Kerasine is the other well-characterized cerebroside. It seems to have the same structure as phrenosin, except that it contains the saturated lignoceric acid, $C_{24}H_{48}O_2$. These two cerebrosides are supposed to be soluble in alcohol but insoluble in petroleum ether, according to Backlin, who groups them together as "saturated cerebrosides."

Backlin lists two other cerebrosides, which he classifies as "unsaturated cerebrosides." The first is nervon, with a structure like the two cerebrosides just described. Its specific acid is nervonic acid, $C_{24}H_{46}O_2$ (43) (46). The other unsaturated cerebroside has no name. It will be referred to here as "klenkosin." According to Klenk (44), its specific acid is "Klenk's acid," an unsaturated hydroxy acid, $C_{24}H_{46}O_3$. According to Backlin, Fränkel has found the unsaturated cerebrosides to be soluble in both petroleum ether and alcohol.

The appearance of cerebrosides in the brain tissue is closely identified with medullation of the nerve fibers. Although medullation begins in the fourth month of fetal life in the human, the period of most rapid medullation is early in post-natal life. Correspondingly, Mendel and Leavenworth (84) found the cerebroside content of the brains of embryonic pigs to be zero, and according to them Raske obtained the same result with fetal calf brains.

Backlin (3) found a very low cerebroside content in the brains of newly-born rabbits. Nearly all workers with this problem have obtained results showing rapid development of cerebrosides in early post-natal life. Koch and Koch (62) found that the greatest increase in the cerebroside content of albino rat brains comes at a period of twenty to forty days after birth. There may be a correlation between the development of the galactose-containing cerebrosides with the consumption of the lactose (hence galactose) contained in milk.

The function of the myelin sheath of nerve fibers is not known. It has been suggested that the sheath is for insulation of the electrical nerve impulses of the nerve fiber or for nutrition of the fiber.

The sterols of the brain are cholesterol, ergosterol, and possibly isocholesterol. Of these, only cholesterol appears in important amounts. Koch's analysis shows that it makes up 9.22 per cent of the dry matter of adult human brains, according to Mathews. Cholesterol is a solid alcohol of the formula $C_{27}H_{46}O$, soluble in various hydrocarbons, chloroform, ether, carbon tetrachloride, acetone, and hot alcohol. Mathews gives the structural formula put forward by Windaus and the one by Wieland. Both formulae contain condensed cyclo hexane rings and a cyclo pentane ring and

a hydroxy group, but there is not much similarity beyond that. The structure of cholesterol may be considered as unknown. No suggestion has been made as to the function of cholesterol in brain tissue.

The sulfatides are the least known of the brain lipins. They occur in only small amounts in the brain tissues. There is not as much agreement on the nature of the sulfatides as there is on the nature of the other brain lipins. Koch, MacLean, Thudichum, Fränkel, and Levene have studied this material. Koch (62) believed it to contain phosphorus. Backlin states that Thudichum (107) thought it to contain phosphorus. Fränkel and his co-workers (30) (31) call it a phosphorus-sulfatide. Levene (69) believes the sulfatide material to be free of phosphorus. According to MacLean (81), the sulfur-containing lipin of Levene was not well enough characterized to be classified as a chemical individual. Fränkel's compound contained 1.58 per cent sulfur, and contained the cerebronic acid radicle. Thudichum's material contained 3 per cent sulfur. Koch believed the sulfatide to be a union of cerebroside and phosphatide through the sulfate radicle. He gave its constitution as 2 per cent sulfur, 42.9 per cent cerebroside, and 51.2 per cent phosphatide. Backlin says that sulfatides should contain phrenosinic acid and hydroxy capric acid.

It is uncertain whether or not the brain lipins isolated exist as such in the living brain. The procedures of isolation may cause rearrangement of structure to take place, and may also disintegrate compounds of a highly unstable and reactive nature. That the brain contains highly reactive materials is indicated by the fact that drugs such as cyanide tend to accumulate in the nervous tissue. The metabolism of the brain seems to be high, for it consumes much oxygen from the blood, and it retains its weight during starvation, a phenomenon characteristic of tissues having high metabolism. These suggestions point toward a highly complex nervous tissue with a constitution differing somewhat from the substances isolated from the dead tissue. Rosenheim's study (97), however, led him to believe that cerebrosides, at least, exist preformed in the brain. It is still possible, of course, that they make up only part of the functional substance of living nervous tissue.

THE METHODS OF BRAIN LIPIN ANALYSIS

The methods of analysis of brain lipins may be divided into two primary divisions, the macro and the micro. The earlier workers conducted their researches by the macro method; the present trend is toward the micro determination.

Backlin gives three general types of procedure which

are followed in either macro or micro methods, once the total lipids are isolated from the nervous tissue. The first is the precipitation of the various lipins with metal salts, such as lead, cadmium, and platinum salts. This method was used largely by Thudichum, who separated many different "constituents" from the brain by this procedure, and who described the salts of the lipins. These are reported in some detail by Mathews. The second method is that of evaporating the total lipin extract and determining the various fractions by extractions with a variety of solvents. This method depends on the differences in solubility of the fractions in such solvents as chloroform, pyridine, acetone, alcohol, ligroin, and diethyl ether. Mathews (83) describes a procedure of this type in his laboratory directions for the qualitative analysis of brain lipins. It is difficult to obtain well-characterized compounds in this way. The third method consists of determining the amounts of phosphorus, or nitrogen, or sugar present in the various fractions and then calculating the amounts of the different lipin compounds present, basing these calculations on the known constitutions of the compounds assumed to be present. This method requires that the solubility behavior and constitution of the compounds be known, and usually necessitates a previous hydrolysis of the individual brain lipin group being analyzed. Nearly all

quantitative methods represent a combination of the second and third procedures.

Backlin states that the earliest publication on quantitative brain lipin analysis was that of Petrowsky (92) in 1873. Petrowsky investigated the lipins of the brain cortex, using the macro methods. He gave values for lecithin, "cholesterol and fat," and cerebrin.

In 1884, Thudichum (107) published his book on the chemical constitution of the brain. His method was that of precipitating the lipins as metal salts and then determining the salts by gravimetric analysis. Although Thudichum's work was later found to be incorrect in some respects, it has influenced nearly all later investigations because of the nomenclature set up and the various compounds described. Mathews gives some of the results published by Thudichum, who worked on human brain material.

Waldemar Koch presented his macro method in 1909 (58) (61) (64) (66). It consists, in short, of an extraction of brain tissue by hot alcohol, evaporation of the extract to constant weight and subsequent emulsification of the residue in water containing a little chloroform and hydrochloric acid. The lipins thus precipitated in the course of a week are filtered out, dissolved in alcohol; and phosphorus, nitrogen, and sugar are determined in aliquots of the solution. Backlin states that this method was used by

Koch and Mann (63) (65) for the study of the brain composition of sick and healthy individuals with special reference to dementia praecox and paralysis. Koch and Koch used the method in 1913 for investigation of the brain lipins of white mice during their development (62). Koch used it in 1913 to compare the brains of newly-born white rats with those of fetal pigs (53). Koch and Voegtlin in 1916 undertook a study of the relation between a restricted vegetable diet and brain composition, and a study of the relation between pellagra and brain composition by use of Koch's method. Their results are given in a lengthy publication (55). Koch and Riddle used the method for studying the brains of normal and ataxic pigeons (54).

According to Backlin, in 1909 Frankel presented a macro method for the determination of brain lipins (24). It consists of a fractional extraction of brain lipins and subsequent isolation, either as cadmium salts or as the individual lipins themselves. Backlin says the method has been used by Frankel and his co-workers on a study of human brains (27) (32), by Bergamini in a study of brains from children having diseases with cerebral symptoms (7), by Pighini in the study of paralysis (93) (94) (95), and by Gralka and Pogorschelsky in a study of diseased brains of children (37).

In a period around 1911 to 1913, Smith and Mair presented a macro method for brain lipin analysis (103). In this method, the brain material is first fixed in formalin, then dried out to constant weight, and extracted with boiling chloroform. A portion of the extract is treated with barium hydroxide solution which saponifies the phosphatides but not the cerebrosides. The material is then neutralized with glacial acetic acid and evaporated to dryness. The residue is extracted with boiling acetone, which removes the cerebrosides. On cooling the acetone, the cerebrosides are precipitated. They are then determined by filtering and weighing. The acetone filtrate is used for determining cholesterol by the digitonin method. The phosphatides are determined as phosphorus in the chloroform solution. Smith and Mair have used this method for the determination of the lipins of human brains (103), and for the determination of the lipin constitution of the brains of growing puppies (104). The wisdom of a formalin fixation for brain material has been questioned by other workers (41) (110).

MacArthur in 1919 (76) published his method for the determination of the chemical constitution of animal tissues. The method is based on the procedure of his teacher, Waldemar Koch. Briefly, the method consists of obtaining the lipin constituents by extracting the tissues which have been fixed in alcohol with ether, chloroform,

water, and hot alcohol. The combined extracts are evaporated down to constant weight; the residue consists of lipins and extractives. The residue is carefully emulsified in water, and the lipins are precipitated by adding hydrochloric acid. The precipitated lipins are washed several times with acidulated water by centrifuging. The water washings and the original aqueous phase of the emulsion contain the extractives. The weight of the extractives is found by evaporating down to constant weight a portion of the extractive solution. The weight of the extractives subtracted from the weight of the original extractive residue gives the weight of the lipins. The lipins are dissolved in absolute alcohol containing a little chloroform. For the hydrolysis, a portion of the lipin solution is evaporated to dryness in a small flask, dilute hydrochloric acid is added, and the contents are refluxed for thirty hours. The liquid is then removed from the flask, and the residue in the flask is refluxed for ten hours with additional dilute hydrochloric acid. The hydrolysis liquid is added to the first portion obtained. The hydrolysis filtrate may now be analyzed for such hydrolysis products as sugar, phosphoric acid, and amines.

Backlin states that in 1929 Weil made a macro determination of brain lipins (109) by working through the procedure sketched by MacLean and MacLean (81). This pro-

cedure is based on the solubility behavior of the lipins. Weil came to the conclusion that the procedure is of no value.

These macro methods all possess several outstanding disadvantages: (1) They require a comparatively large amount of material. In one of his analyses, Koch used one hundred rat brains; Smith and Mair used several puppy brains for one determination. (2) They require a rather long time for an analysis. The total time often involves several weeks or months. (3) They require rather large amounts of reagents.

It follows from these points that it is impossible readily to run a large number of determinations or to make determinations on individual animals by using the macro methods. The micro methods obviate such disadvantages.

The first micro method for brain lipin determination was published by Gorodissky in 1925 (34). Gorodissky makes use of the technique for lipin extraction worked out by Bang for his blood lipin determination (5). It consists of spreading thirty to sixty milligrams of the tissue upon fat-free strips of filter paper. The strips are dried in a vacuum dessicator and are then ready for extraction. Gorodissky uses Fränkel's procedure of successive extractions with boiling acetone, petroleum ether, and boiling alcohol. The extracts are then used for phosphorus determinations,

oxidation values, etc. Several values for lipin constituents are determined by difference. Gorodissky has used her method for a study of the chemical topography of the human brain (34) (35), Tscherkes and Gorodissky have used it for studying the influence of several narcotics on the brain lipins (108), and Serejski for a study of narcosis (101) (102).

A micro method for determination of cerebrosides was described by Kimmelstiel in 1929 (39). Kimmelstiel makes use of the Hagedorn-Jensen method of sugar determination, with a modification of the Noll method (89) of hydrolysis. By making a preliminary determination of the reducing power of his lipin extract before hydrolysis, Kimmelstiel has sought to eliminate an error due to reducing substances other than the sugar of the cerebroside. The method has been used by Jungmann and Kimmelstiel for an investigation of the origin of the lactic acid of the central nervous system (38), by Kimmelstiel for investigation of autolysis of brain lipins after the death of the animal (40), and by Kimmelstiel for a study of the effect of formalin fixation on the lipin compounds of the brain (41).

Mitolo (86) cites a micro method for the determination of phosphatides by use of the nephelometer. The method was published in 1930 by Crema (19).

In 1933 Mitolo published an outline for the micro de-

termination of lipins in nervous tissue (86). The method is in part similar to that of Backlin. It calls for fractional extraction by several solvents. Unfortunately, Mitolo did not discuss his experience with the method or give any results obtained by the use of it.

EXPERIMENTAL PART

It was desired to know the distribution of the constituents of the brain lipins of normal rats of varying ages. It was also desired to know the amount of variation to be expected among individual rats. Koch and Koch (62) had done some work on the brain lipins of rats, but the method used by them requires considerable time and determinations on the brain lipins of individual rats would hardly be possible by their macro method.

After considering the analytical methods available, the micro method of Backlin (3) was chosen, for it afforded a rapid and apparently precise method of determining various lipin constituents. The lipin fractions obtained by this method are: (1) cholesterol, (2) unsaturated cerebrosides, (3) unsaturated phosphatides, (4) saturated phosphatides, and (5) saturated cerebrosides. The nomenclature used for this description of the fraction was discussed in the section dealing with the chemical constitution of ner-

vous tissue. Since any method of brain lipin analysis is somewhat empirical, the values obtained in normal individuals is necessary as a preliminary to further studies on the relation of nutrition, or of pathological conditions, to the chemical constitution of the brain. Consequently, it was desired to know the distribution of the brain lipins of normal rats, as found by the Backlin method. These values could then be used as a comparison for further studies.

A series of experiments on normal albino rats between the ages of 10 and 150 days was undertaken. The procedure used was essentially that given by Backlin. A few variations were introduced; these will be discussed during the description of the method.

The Backlin method is as follows: The fresh brain tissue, freed of any large membranes or blood vessels, is passed through ordinary glass syringes of 2 cc. to 10 cc. capacity. These syringes contain at their lower ends sieves made of copper screen and so cut as to fit snugly inside the hollow syringe cylinder. The material is passed through alternate syringes until the material is in a homogeneous, pasty mass. This stage is reached by the fifteenth sieving. For a single determination, 60 to 100 mg. of this pasty brain material are spread upon two fat-free

filter paper strips measuring about 17 by 28 mm. The strips are quickly weighed to 0.1 mg. They are then placed in a vacuum dessicator and dried for an hour.

After the drying, the strips are ready for the lipin separation procedure. For a single determination, four ordinary test tubes of about 25 cc. capacity are needed; they are numbered 1, 2, 3, and 4. Four separatory funnels, capable of being centrifuged, are needed, and they are numbered 1, 2, 3, and 4. The procedure for fractionation and hydrolysis of the lipins is given in table 1. The table presents in a condensed form the procedure as described by Backlin, and was worked out after considerable experience with the method. It is so arranged that the complete separation of the lipins can be effected with a minimum loss of time. It has been found helpful to have the table at the side of the analyst as he is working. Occasional reference to it is usually necessary for one not thoroughly acquainted with the rather detailed procedure.

Tables 2 and 3 give an outline of the theoretical basis for the method. It will be seen that the method is based principally on two phenomena. The first phenomenon is that extraction with petroleum ether removes cholesterol, the unsaturated phosphatides, and the unsaturated cerebrosides; extraction with absolute alcohol removes the saturated phosphatides and the saturated cerebrosides. Since a

Table 1. Procedure of Analysis by Backlin Method

1	2 3	T1	Extract dried strip in petroleum ether (Pet) 40 min.	
1	2 5	T2	Further extract strip in boiling EtOH 60 min.	
1	3 4	T1	Evaporate extract 1 nearly to dryness (5 min.)	
3	4 6	T1	To residue in T1 add 5 cc dry EtOH + 0.2 cc 15% NaOH and dry on water bath with added salt. (30 min.)	
2	5 9	T2	To extract in T2 add 0.2 cc 15% NaOH and dry on water bath with added salt. (30 min.)	
4	6 7	T1 Sep.1	To residue in T1 add 3 cc 5% NaOH, shake well, and pour into Sep.1. Extract the T 5 min. with 10 cc Pet.	
6	7 8	T1 Sep.1	Transfer Pet from T1 to Sep.1, shake 3 min., drain lower layer back into T1 without breaking salty surface, and centrifuge Sep. 5 min.	
7	8 10 12	Sep.1 T1 T3	From Sep.1 pour off Pet layer into T3 and evaporate on water bath nearly to dryness. (5 min.) To Sep. add 2 cc 20% H_2SO_4 and then 3 cc H_2O. Shake Sep. and draw off contents into T1. Return soln. to Sep. and extract T1 5 min. with 10 cc Pet.	
5	9 11	T2 Sep.2	To residue in T2, add 3 cc H_2O, shake well, pour into Sep. 2 and rinse T first with 2 cc and then with 1 cc of 20% H_2SO_4 and drain into Sep.2. Extract T2 5 min. with 10 cc Pet.	
8	10 14	T3	To nearly dry T3 add 5 cc 20% H_2SO_4 and heat 30 min. on water bath.	
9	11 13	T2	Transfer Pet from T2 to Sep.2, shake 3 min. and alternately drain and centrifuge until ether layer is clear and water layer is returned to T2.	
8	12	Sep.1 T1 Ic	To Sep.1 add Pet from T1, shake 3 min., settle, draw off lower layer into T1 and pour off Pet layer into tube Ic.	
11	13 15	Sep.2 IIa T2	From Sep.2 pour off Pet layer into tube IIa. Add 2 cc 20% H_2SO_4 and then shake. Draw off liquor into T2 and heat 30 min. on boiling water bath.	
10	14 16	T3 Sep.3	Cool T3, transfer contents to Sep.3, treat with 6.5 cc 25% NaOH and cool. Extract T 10 min. with 10 cc. Pet.	
13	15	T2 Sep.2 IIb	Cool T2, transfer contents to Sep.2, extract T 5 min. with 10 cc Pet, and transfer Pet to Sep. Shake 3 min., settle, draw off lower layer into T2, and pour Pet into tube IIb.	
14	16 17 18	T3 Sep.3 T4	Transfer Pet from T3 to Sep.3, shake 3 min., draw off lower layer into T3, settle or centrifuge Pet layer, pour off into T4, and evaporate nearly to dryness. Wash Sep.3 with 2 cc 20% H_2SO_4, draw off into T3, return to Sep. and then extract T3 5 min. with 10 cc Pet.	
16	17 19	T4 Sep.4	Treat residue in T4 with 1 cc 20% H_2SO_4 + 3 cc H_2O, warm 3-4 min. in water bath, transfer to Sep.4 and extract the T 5 min with 10 cc Pet.	
16	18	T3 Sep.3 Ib	Transfer Pet from T3 to Sep.3, shake 3 min., draw off lower layer, and pour Pet layer into tube Ib.	
17	19	T4 Sep.4 Ia	Transfer Pet from T4 to Sep.4, shake 3 min., draw off lower layer, and pour Pet layer into tube Ia.	

Note:

The column of underlined figures numbers the steps of the procedure.

The column immediately to the right of the column of underlined figures indicates the steps to which the material goes next.

The column at the extreme left indicates the steps from which the material comes.

The column at the extreme right indicates the marked glassware used.

Explanation of Symbols used in this Table

Pet = Petroleum ether

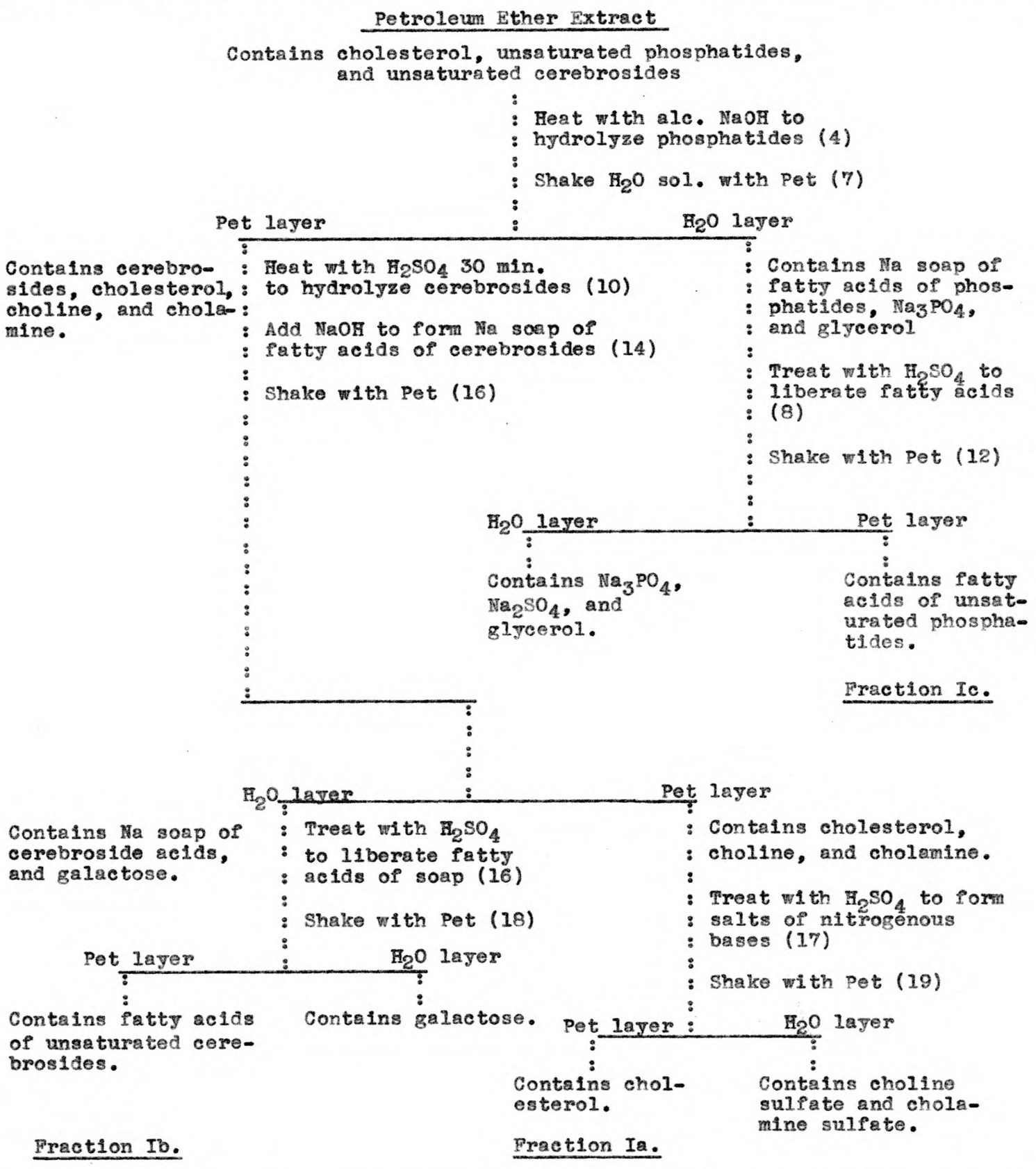
EtOH = Absolute ethyl alcohol

T = Test tube

Sep = Separatory funnel

Ia = Test tube for cholesterol extract
Ib = Test tube for extract of fatty acids from unsaturated cerebrosides
Ic = Test tube for extract of fatty acids from unsaturated phosphatides
IIa = Test tube for extract of fatty acids from saturated phosphatides
IIb = Test tube for extract of fatty acids from saturated cerebrosides.

Table 2. Theory of Analysis of Petroleum Ether Extract



Note:

Pet = Petroleum ether.

The numbers in parentheses refer to the steps of the Procedure given in Table 1.

Table 3. Theory of Analysis of Alcoholic Extract

Alcoholic Extract

Contains saturated phosphatides and saturated cerebrosides

Hydrolyze phosphatides with alcoholic NaOH; Na soaps of fatty acids of phosphatides are formed (5).

Treat with H_2SO_4 to liberate fatty acids from the Na soaps (9).

Shake with Pet (11).

H_2O layer

Contains cerebrosides, phosphoric acid, sphingosine sulfate, and choline sulfate.

Heat with H_2SO_4 to hydrolyze cerebrosides (13).

Shake with Pet (15)

H_2O layer

Contains phosphoric acid, sphingosine sulfate, choline sulfate, and galactose.

Pet layer

Contains fatty acids of saturated cerebrosides.

Fraction IIb

Pet layer

Contains fatty acids of saturated phosphatides.

Fraction IIa

Note:

Pet = Petroleum ether

Numbers in parentheses refer to steps of procedure given in Table 1.

better separation is obtained between the unsaturated and the saturated phosphatides with a low-boiling petroleum ether, normal pentane was substituted for petroleum ether in the first extraction of the procedure.

The second phenomenon is that treatment of the lipins with alkali hydrolyzes the phosphatides but not the cerebrosides (if not too severe a treatment is given). The cerebrosides may be hydrolyzed by heating with acids.

With the exception of cholesterol, it is the fatty acids of the various fractions which are measured. The amount of lipin present may be calculated, if desired, from the values for the fatty acid fractions. The fatty acids and the cholesterol are finally obtained in 10 cc. extracts of petroleum ether. The various fractions are placed in small pyrex test tubes, numbered Ia, Ib, Ic, IIa, and IIb. The extract in Ia contains the cholesterol, that in Ib the unsaturated cerebrosides, that in Ic the unsaturated phosphatides, that in IIa the saturated phosphatides, and that in IIb the saturated cerebrosides.

The petroleum ether is evaporated off and the tubes are weighed on a micro balance. Since the method of analysis is such that salts from the aqueous phase of the extraction funnel are often present in the tube along with the fatty acids, the tube is next carefully rinsed out with petroleum ether. This removes the fatty acids but not the

salts. The tube is again weighed and the difference between the two weights is taken as the weight of the fatty acids.

This method of determining the amount of fatty acids obtained is quite different from that of Backlin (2). Backlin oxidizes the material in the tube by use of a silver dichromate-sulfuric acid reagent. The carbon dioxide formed is absorbed in alkali solution. The alkali is then treated with excess acid, liberating the carbon dioxide, which is determined by volumetric method. The amount of fatty acid originally present may be found by suitable calculations.

The method used here for determining the values of the lipin fractions is believed to be superior to that of Backlin for three reasons: (1) It may be carried out more rapidly. (2) It does not require the expensive reagents. (3) It eliminates the possibility of measuring organic salts which might be present in the tube. Backlin would measure the carbon dioxide coming from almost any organic compound likely to be present with the fatty acids.

The value for total solids is obtained by preparing weighed strips of filter paper with the brain material spread on them; the paper strips are the same sort as used in the extraction procedure. The strips with the brain material are weighed. Then they are dried for two hours in

an oven at 100°C. They are removed and allowed to cool in a dessicator. They are weighed again, and the final weight of the material on the strips is the weight of the total solids.

The method of Backlin with the modifications described was used to determine the distribution of brain lipins in a number of albino rats of varying ages. The amountsof deviation between different individuals of the same age groups were calculated. The results are summarized in table 4.

Backlin's method was worked out by him on a theoretical basis from results obtained in macro studies of brain lipins. He does not give any data obtained by putting a known sample through his procedure. Since Backlin's method was found to be useful in brain analysis, it was deemed desirable to check our results by some other method. The most direct check would be that of determining the amount of some hydrolysis product present in the aqueous residues obtained in the analysis. It was especially desired to obtain a check on the cerebroside portion of the samples. Backlin states that the aqueous residues from these fractions contain the free galactose from the hydrolyzed cerebrosides. Tables 2 and 3 show the residues which should contain the galactose. Calculation showed that, according to the amounts of fatty acid obtained from cerebroside fractions, there should be sufficient galactose present to

Table 4. Distribution of Brain Lipins of Normal Rats.

Age of animals : 10 days : 15 days : 20 days : 35 days : 40 days : 150 days No. of animals : 5 x 2 : 4 : 4 : 4 : 7 : 17						
Constituent value:						
Ia = Cholesterol						
Average %	.535	.862	1.37	1.30	1.88	1.65
Dev. of dupl. Ave.	.044	.082	.08	.08	.22	.18
Variation, Max. Ind.	.143	.297	.65	.24	1.30	2.02
Ib = Acids from unsaturated cerebrosides (Klenkosin + Nervon)						
Average %	.087	.149	.153	.174	.235	.505
Deviation	.015	.015	.020	.023	.047	.217
Variation	.096	.106	.196	.054	.285	.582
Ic = Acids from unsaturated phosphatides (Lecithin + Cephalin)						
Average %	.944	1.65	2.17	2.50	2.33	1.58
Deviation	.027	.05	.10	.25	.30	.23
Variation	.109	.27	.34	.24	.61	1.69
IIa = Acids from saturated phosphatides (Sphingomyelin)						
Average %	.96	1.29	1.98	2.54	2.20	2.33
Deviation	.07	.18	.24	.11	.20	.37
Variation	.25	.44	.78	.50	1.53	2.08
IIb = Acids from saturated cerebrosides (Phrenosin + Kerasin)						
Average %	.294	.172	.260	.470	.380	1.011
Deviation	.113	.070	.078	.130	.038	.246
Variation	.284	.212	.257	.182	.305	1.51
III = Total solids						
Average %	12.2	13.6	16.6	19.3	20.3	21.4
Deviation	.3	.5	.4	.4	.6	.5
Variation	2.1	4.7	4.4	3.1	2.5	3.4
IV = Brain weight						
Average grams	.697	1.024	1.323	1.447	1.472	1.676
Variation	.379	.325	.267	.355	.251	.614
V = Body weight						
Average grams	11.9	19.2	32.8	65.1	74.5	238.9
Variation	11.2	16.8	10.0	12.7	39.5	96.5

Lines marked "Average" indicate average of all animals in age group for which duplicate analyses are available.

Lines marked "Deviation" indicate the average deviation of single determinations from the mean.

Lines marked "Variation" indicate difference between average for high and low animal of each group.

enable a colorimetric determination to be made. The amount of galactose indicated by these calculations varied from 0.2 mg. to 1.2 mg. for one aqueous residue. Benedict's blood sugar method was chosen as being the one best suited for an analysis of the sugar in the residues, for it is said to have a selective action toward sugars.

The first measurements were tried on portions of the aqueous residues, treating them as though they were samples of blood filtrate. In these trials, color was obtained with the residues from the unsaturated cerebrosides, but none from the saturated cerebrosides. It was soon discovered that the residues from the saturated cerebroside fractions were too acid. When those residues were neutralized and then put through the Benedict procedure, color was obtained. In both cases, however, the amount of color obtained was too small to enable accurate measurements to be made. In addition, not as much color appeared as was expected.

It was decided to evaporate the neutralized solutions down to a 2 cc. volume, which is the volume of blood filtrate specified to be used in the Benedict blood sugar method. This 2 cc. sample would contain all the galactose present in one aqueous residue. It was discovered upon trying this that there was too much salt present to allow such a procedure to be used. It appeared that if two aqueous residues were combined and evaporated to a small volume,

better measurements might be obtained. When this was tried, an unmistakable blue color, characteristic of the method, was obtained. Again, however, the amount of color developed was too small to afford a satisfactory determination.

It was suggested that the evaporating of the residues on the water bath might be oxidizing the sugar. To prevent such an oxidation from readily occurring, the combined residues were neutralized carefully, buffered with ammonium acetate, and evaporated to a smaller volume. Some blue color was obtained with this method, but not enough for a good determination.

It was decided to attempt to obtain the sugar from the residues in an aqueous solution free of all the other products of the hydrolysis. Accordingly, two aqueous residues from cerebroside fractions were combined in an evaporating dish, and neutralized carefully. They were then evaporated in the cold with the aid of an electric fan. Occasional stirring of the wet salt mixture obtained upon evaporation and continued use of the electric fan finally resulted in the obtaining of dry salt residues. This process took about forty hours. The dried salt residues were then extracted carefully with six successive portions of 80 per cent ethyl alcohol. This should remove the sugar and yet not take up much of the inorganic salts present.

The alcohol solution was then evaporated to dryness and taken up in distilled water. The sugar measurements made by this process were improved, but the color obtained in the final solution was a blue-green one instead of the desired blue one. Here, also, the intensity of color present did not afford accurate measurements.

It was decided that the color was possibly being contaminated by the presence of nitrogenous bases obtained on the original hydrolysis. The procedure just outlined was repeated, but just before taking up the alcohol extract residue in water, it was extracted with various organic solvents such as chloroform, pentane, and absolute alcohol. These solvents should remove the free nitrogenous compounds likely to be present, sphingosine, choline, and cholamine (kolamine). The color improved with this treatment, but it still was not satisfactory.

The procedure as given above was repeated with more aqueous residues, this time adding a known amount of galactose at various steps in the system. The sugar was added to some residues just before neutralization and to others just after neutralization. When the residues were put through the refined procedure, not all the added galactose could be determined by the method. And even when the galactose was added after the extraction with the organic solvents, there was a definite decrease in the sugar values

obtained by use of the colorimeter. The attempt to get a direct check on the cerebrosides was abandoned when it was found that the added galactose could not be determined in the aqueous residues. The best results obtained in the study of the sugar from the cerebroside hydrolysis were about one-tenth of the theoretical values obtained by calculation from the fatty acids determined from the same fractions.

The attempts to get a direct check on the cerebroside fractions by use of the hydrolysis residues having proved to be unsatisfactory, it was concluded that the next best check on those fractions would be to compare them with the values obtained by an entirely different method. The method of MacArthur (76) was selected as being one of the best of the macro methods. It has been described briefly in the section on the methods of brain lipin analysis. MacArthur's method is based upon that of his teacher, Waldemar Koch (61).

The hydrolysis filtrate obtained in the MacArthur method may be tested for reducing sugar, which would enable the total cerebrosides present to be estimated. MacArthur specifies the picric acid method for determination of reducing sugar in his hydrolysis filtrate. In this method, 2 cc. of the filtrate are neutralized in a test tube. Then 2 cc. of a saturated solution of picric acid and 2 cc. of

20 per cent sodium carbonate solution are added. This mixture is heated for an hour in boiling water, after which the unknown is compared in a colorimeter with a known sugar solution which has received identical treatment.

However, it was found upon trial of the picric acid method on the hydrolysis filtrate of the MacArthur method that the picric acid procedure gave abnormally high values for cerebroside content. The values obtained indicated that the cerebrosides made up half of the total lipins. This is clearly too high a value.

The Benedict blood sugar method was then tried on the hydrolysis filtrate. This procedure gave values which appear to be much nearer the truth.

The MacArthur method, combined with the Benedict blood sugar method, was used to check the cerebroside values obtained by the Backlin method. Four different samples of albino rat brains were used in the comparison. A small portion of each sample was used for a quadruplicate determination by the Backlin method and the remainder of the sample was used in the MacArthur method. The comparison of cerebroside values was obtained by the use of the two different methods. The results are summarized in table 5.

Table 5. A Comparison of the Cerebroside Values Obtained by Use of the MacArthur and Backlin Methods of Brain Lipin Analysis

Sample	Per cent Cerebroside in Lipin		Per cent Cerebroside in Total Sample	
	MacArthur	Backlin	MacArthur	Backlin
I	7.47	4.40	0.616	0.56
II	7.86	6.90	0.586	0.75
III	6.14	----	0.888	----
IV	11.85	32.5	1.20	3.56

DISCUSSION OF RESULTS

Table 4 gives the distribution of brain lipins of young albino rats of various ages, according to the Backlin method of analysis. It is evident that the saturated phosphatides, or the sphingomyelins, make up the greater part of the brain lipins. This is in agreement with general observations. The lecithins and the cephalins, cholesterol, and the phrenosins and the kerasins follow in amount present in the order named. The less familiar compounds, nervon and "klenkosin," make up only a small proportion of the brain lipins.

It is evident that the increase in cerebrosides shortly after birth is no greater than the increase of phosphatides or cholesterol. This is in agreement with the work

of Koch and Koch (62). On the basis of this determination, no inference can be drawn that the cerebrosides are to be singled out for correlation with medullation. As the cerebrosides make up more of the white matter of brain than they do the grey matter, they may be supposed to increase because of medullation.

Measurable amounts of cerebrosides were found in the ten day old rats; Koch and Koch did not attempt to determine cerebrosides in rats of that age.

It is significant that a variation in brain lipins between individuals of the same age was found. Indeed, variation was found in the brain lipins of litter-mates. This would provide a basis for further studies on the relation of inheritance, nutrition, etc., to the chemical constitution of the brain.

The experiments with the aqueous residues of the cerebroside fractions are open to several interpretations. It may be that the sugars from the cerebroside hydrolysis are not present in the residues in which they theoretically should occur. It may be that there are interfering substances in the residues which unite or react with the sugar in such a way that the reducing power is lowered; the experiments with the added galactose point in this direction. It is possible that, in spite of the care exercised in the sugar extraction, the sugars were oxidized. The first hy-

drolysis of the phosphatides with alkali solution may effect the cerebrosides, although Smith and Mair (103) and Backlin (3) claim that there is no such effect. Satisfactory measurements of the galactose said to be present could not be secured.

The difference in the amount of cerebroside determined by the Backlin and by the MacArthur methods is to be expected. When dealing with the rather poorly defined constituents of brain lipin material, it is unlikely that two very different methods will give the same result. In table 5, samples I and II give the same order of cerebroside value which compared by the two methods. Sample IV does not give the same order of value, although with both methods the variation between sample IV and samples I and II is in the same direction, and unmistakeably indicates a difference in constitution between sample IV and samples I and II.

It is reasonable to conclude that the two methods measure somewhat different constituents, but that there is a similarity between these constituents. Both include a sort of material referred to as cerebroside, which contains a substituent having a reducing power similar to that of a reducing sugar. This substance may be galactose.

SUMMARY

The distribution of the brain lipins of normal young

rats has been found by use of a modification of the Backlin method of brain lipin analysis. The results indicate a variation in the brain lipins of individuals of the same age, and even of the same litter. The data presented may be used as a basis for comparison with values obtained by other methods and for a comparison of subsequent work with the Backlin method.

The aqueous residues of the Backlin method were tested for galactose from the cerebroside fraction. Although there was clear evidence of a reducing compound, probably a reducing sugar, no satisfactory measurements could be obtained and the amount of sugar apparently present was considerably below the theoretical values.

A comparison of the cerebroside values by use of the Backlin method and of the MacArthur method of tissue analysis was made. The values obtained indicate that the two methods measure similar compounds, but not exactly the same compounds. It appears that the constituents of brain lipin material can not be sharply differentiated, and that the functioning nervous tissue may be composed of a highly reactive complex substance which yields the fractions obtained upon analysis.

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