[CONTRIBUTION FROM THE BIOCHEMISTRY LABORATORY OF THE UNIVERSITY OF ILLINOIS.]

BRAIN CEPHALIN. II. FATTY ACIDS.

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Received April 1, 1916.

As has been stated in the previous article of this series,[1] there is some uncertainty as to what fatty acids are present in the cephalin molecule and considerable uncertainty as to the amounts of each.

Preparation.—In this work preparations have been made in several ways, some old, some new, to see whether there are consistent differences in the molecule depending on the method of preparation.

Cephalins (1), (2), (3) and (4) were prepared and purified as described in the previous paper. Another preparation, purified like cephalin (4), was further purified by dissolving in chloroform and precipitating with twice the solution's volume of absolute alcohol. The object of this treatment was to remove any trace of sphengomyelin that might still contaminate the product. The desiccated material was then emulsified in water and precipitated by an equal volume of acetone. The precipitate was further washed with acetone, placed in a vacuum desiccator and dried, then taken up in ether, centrifuged, and precipitated by acetone and dried. This will be referred to as cephalin (8). It is a light yellow powdery product, similar in appearance to cephalin (4).

Percentage of Total Fatty Acids in Cephalin.—To determine the percentage of total fatty acids in cephalin, one gram of the cephalin was hydrolyzed for 20 hours in a 1% hydrochloric acid solution. The fatty acid residue, after filtration, was dissolved in ether, filtered, and carefully evaporated to dryness in a weighed dish. It was dried over sulfuric acid till constant weight was obtained. By this method cephalin (1) (HCl purified) gave 0.670 g. of fatty acids. One gram of cephalin (4) gave 0.652 g. The fatty acids from another sample of cephalin (1) weighed 0.66 g. From this data it seems that 66% of cephalin is in the form of fatty acids. It is likely that these values are slightly high because there is a small amount of an unhydrolyzable substance that would be included in the total fatty acids.

Quantitative Study.

Four grams (usually) of the cephalin were dissolved in 200 cc. of a 2% water potassium hydroxide solution. This was boiled for 20 hours under a reflux condenser, cooled and acidified with acetic acid. The fatty acids were filtered rapidly, washed three times with distilled water, then dissolved in dilute potassium hydroxide. The solution was now neutralized with acetic acid and an equal volume (about 150 cc.) of a 5% lead acetate solution added. The lead soaps were filtered through a

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[1] MacArthur, THIS JOURNAL, 36, 2397 (1914).

hardened filter, washed three times, and dried with filter paper and then in a vacuum desiccator. The comparatively dry soaps were carefully placed in a flask and refluxed with about 150 cc. of freshly distilled ether for several hours until all the particles were finely divided. The contents of the flask were cooled and either filtered or centrifuged and washed with small amounts of ether. In some experiments better results were obtained by allowing this solution to stand a few hours in a tall cylinder and then siphoning off the clear solution. The residue was washed three times.

The saturated insoluble lead soaps were decomposed either by passing H_2S through the hot benzene solution, or by refluxing the lead soap with an ether and hydrochloric acid solution in the usual manner. Several repetitions of this procedure were necessary. The free acids were evaporated to dryness and weighed. This gave the total saturated acids. The product was then recrystallized several times from alcohol and acetone, and was ready for melting point and molecular-weight determinations and derivatives.

The unsaturated lead soaps were decomposed by shaking the ether solution with several portions of a dilute HCl solution. The ether solution was then washed with water, dried with anhydrous sodium sulfate, and filtered into a flask.

The solution of the unsaturated acids was cooled in ice water, and bromine added in small portions till an excess was present. They were allowed to stand overnight in ice bath in the ice box, then placed in a corked weighed centrifuge tube and the ether-insoluble bromine derivative centrifuged out. It was washed with ether several times, dried and weighed. From a bromine determination on this compound the amount of fatty acid in it could be calculated. The bromine determination also helped to identify the acid.

The ether solution of the ether-soluble bromine derivatives was placed in a separatory funnel and an excess of sodium thiosulfate added. The funnel was thoroughly shaken, allowed to stand, and then the water layer was drawn off. The ether was again treated with sodium thiosulfate. Finally it was washed with several portions of water, dehydrated with anhydrous sodium sulfate, filtered into a weighed beaker and carefully evaporated to dryness. The weight gave the amount of the ether-soluble bromine derivatives.

To separate these the mixture was extracted at room temperature several times with small portions of petroleum ether (b. p. 30–50°). A white insoluble bromide remained. The petroleum ether solution was evaporated carefully to dryness and the residue again treated with small amounts of petroleum ether. A small portion remained undissolved. These two residues were united, dried and weighed. They represent the petroleum-ether-insoluble bromine derivative. From the bromine determination the amount of free fatty acid was determined. This bromine value and the melting point were used to give an idea of the identity of the compound.

The petroleum ether solution was evaporated to dryness in a weighed beaker. This gave the petroleum-ether-soluble bromine derivative. As a rule this weighed residue was dissolved in acetone. There was a small amount of an acetone-insoluble saturated compound containing 1.3% nitrogen left, probably not fatty acid. The solution was evaporated and weighed. This gave the true weight of the liquid dibromide. From the amount of bromine in it the amount of the fatty acid could be calculated.

By the above procedure the following data were obtained:

TABLE I.

2 g. sheep cephalin (8). Calculated weight of acids = 1.32 g.

	Wt. of bromide.	Wt. of acid.	% of total acids.
Stearic acid	..	0.43	32.6
Oleic acid	1.14	0.73	55.4
Cephalinic acid	0.24	0.09	7.0
Clupanodonic acid	0.17	0.05	3.9
		1.30	98.9

6 g. sheep cephalin (4). Calculated weight of acids = 3.96 g.

	Wt. of bromide.	Wt. of acid.	% of total acids.
Stearic acid	..	1.21	30.6
Oleic acid	3.41	2.18	55.5
Cephalinic acid	0.64	0.26	6.5
Clupanodonic acid	0.49	0.16	4.0
		3.73	96.6

3 g. beef cephalin (4). Calculated weight of acids = 1.98 g.

	Wt. of bromide.	Wt. of acid.	% of total acids.
Stearic acid	..	0.58	29.3
Oleic acid	1.60	1.05	53.0
Cephalinic acid	0.36	0.15	7.5
Clupanodonic acid	0.21	0.07	3.5
		1.85	93.3

In a few analyses the hydrolysis of the cephalin and the solution of the fatty acids in ether were carried out as above described. Instead of separating the saturated from the unsaturated acids, however, the ether solution was brominated and separated as before. The saturated acids will be in with the oleic bromide. The two were weighed together, then from a bromine determination on a fraction of this mixture the amount of oleic bromide and oleic acid could be calculated. The difference between the weight of oleic bromide and that of the mixture was assumed to be stearic acid. From the following data it will be noticed that the agreement is rather close with that of the lead method:

TABLE II.

3 g. sheep cephalin (4). Calculated weight of acids = 1.98 g.

	Wt. of bromide.	Wt. of acid.	% of total acids.
Stearic acid	..	0.55	27.8
Oleic acid	1.75	1.12	56.6
Cephalinic acid	0.45	0.18	9.0
Clupanodonic acid	0.23	0.07	3.5
		1.92	96.9

Discussion of Quantitative Experiments.—A consideration of the data will show that there is no definite relationship between the amounts of the various acids in cephalin. Stearic acid comprises somewhat more than one-fourth of the total fatty acids. According to the generally accepted formula of cephalin, one-half of the cephalin would be present as stearic acid. Oleic acid constitutes about one-half of the total acids. Approximately one-tenth is cephalinic acid, and one-twentieth clupanodonic. It might be thought that one nitrogen constituent was present with each acid, thus giving a mixture of stearyl cephalin, oleyl cephalin, etc. There does not seem to be enough agreement, however, between the amounts of the nitrogenous compounds and the acids to warrant this assumption.

To explain the presence of these acids in such different amounts, it is best to assume that some of the cephalin molecules may have oleic and cephalinic acids, and still others stearic and cephalinic, etc. It is well known that ordinary fats are often thus mixed. It is reasonable, then, to suppose that a highly labile compound like cephalin may have varying fatty acids on any particular molecule. They may even be changing from molecule to molecule in the cephalin mixture.

It is exceedingly difficult to purify cephalin. It is next to impossible to separate the constituents of the mixture, even though some of the supposed cephalin molecules have such different acids as clupanodonic and stearic in them. Because of these facts one may think of cephalin as made up of several constituents more closely associated than a mixture but less so than a simple compound. It may be this power of forming comparatively firm association compounds that partially explains its peculiar importance in brain cell metabolism.

Identification of the Fatty Acids.

To obtain the individual fatty acids or their derivatives for identification, the same procedure was used in most cases as for the quantitative data. For a few experiments, in order to obtain larger amounts of the ether-insoluble and petroleum-ether-insoluble derivatives, 8 g. of the cephalin were hydrolyzed as described and all the free fatty acids dissolved in ether, then brominated and separated according to the above method. In this way the saturated acids went into the ether-soluble bromide fraction, and so did not interfere with the preparations sought.

Stearic Acid.—The somewhat impure saturated acid obtained from sheep cephalin (4) by the quantitative procedure described above gave a molecular weight of 289, a sample of sheep cephalin (1) gave 290.

(4) 0.1096 g. acid	3.7 cc. 0.1 *N* alk.	289 m. w.
(1) 0.2285 g. acid	7.8 cc. 0.1 *N* alk.	290 m. w.

These results were obtained by the usual procedure, dissolving the acid in a definite amount of alcohol and titrating with 0.1 *N* alkali. The number of cc. used in a control on an equal volume of alcohol was subtracted from the fatty acid value.

These impure samples were crystallized twice from alcohol and twice from acetone. The molecular weights then were 284.5 and 284.

(4) 0.2310 g. acid	7.95 cc. 0.1 *N* alk.	284.5 m. w.
(1) 0.2080 g. acid	7.15 cc. 0.1 *N* alk.	284.0 m. w.

This pure acid melted at 69°. Several other samples purified by crystallization from alcohol and acetone gave melting points varying from 68.8° to 69.2°.

The ethyl ester prepared in the usual way had a melting point of 33.4°.

Because the molecular weight of stearic acid is 284, its melting point is 69°, and ethyl stearate melts at 33.5° it may be concluded that stearic acid is the only saturated acid in cephalin in appreciable amounts. It was not determined what impurity present caused the high molecular weight of the impure stearic acid.

Oleic Acid.—The ether-soluble bromide fraction, after removal of the nitrogenous impurity by the latter's insolubility in acetone, was evaporated to dryness, dried over sulfuric acid and a bromine determination made on the heavy yellowish oil in the following way:

About 0.3 g. of the bromine derivative was carefully weighed into a nickel crucible. Ten grams of sodium hydroxide and 3 g. of potassium nitrate and a few drops of water were added. The crucible was covered and carefully heated to avoid spattering. Gradually the flame was raised till a melt was obtained. The heating was continued till the liquid was perfectly clear. It was allowed to cool partially, then was dissolved in water and made slightly acid with nitric acid. One cc. of 10% ferric ammonium sulfate was added and the solution titrated with 0.1 *N* silver nitrate and ammonium thiocyanide.

A few typical determinations out of many on the ether-soluble bromine fraction are given below:

	Bromine.
Sheep cephalin (8) 0.3000 g. bromine compound	36.5%
Sheep cephalin (4) 0.3891 g. bromine compound	37.0%
Sheep cephalin (1) 0.3353 g. bromine compound	34.5%
Beef cephalin (4) 0.3047 g. bromine compound	34.5%

The lowness of a few of the values can easily be accounted for by supposing that a small amount of stearic acid was present. That would be the case if the lead separation had not been complete, and of that it is difficult to be certain.

In order to be sure that this method of determining the bromine content of a fatty acid derivative was entirely correct, the amount of bromine in pure dibromostearic acid, tetrabromostearic acid and hexabromostearic acid was found.

	G.	Obtained. Br.	Theoretical. Br.
Pure dibromostearic acid	0.2941	35.9%	36.2%
Pure tetrabromostearic acid	0.2134	53.2%	53.3%
Pure hexabromostearic acid	0.1070	62.8%	63.3%

It will be noticed that these values agree as well as could be expected with the theoretical bromine values.

The identity of the oleic acid was more firmly established by oxidizing a mixture of the fatty acids of cephalin in the usual way with a permanganate solution. A hydroxy derivative was found that had the properties of dihydroxy stearic acid. It was insoluble in water, somewhat soluble in ether, and readily soluble in hot alcohol.

Cephalinic Acid.—The petroleum-ether-insoluble fraction was purified by dissolving it in ether and filtering to remove any hexa or octa bromine derivatives. The ether solution was evaporated to dryness and the residue recrystallized from petroleum ether.

From the work done on this bromine derivative it is difficult to say what the formula of the compound really is. The bromine values found can be interpreted in several ways.

	Br.
Sheep cephalin (8) 0.2214 g. bromine compound	61.4%
Sheep cephalin (4) 0.2112 g. bromine compound	59.8%
Sheep cephalin (4) 0.2115 g. bromine compound	60.5%
Beef cephalin (4) 0.1178 g. bromine compound	59.0%

Though the bromine derivative from the best samples of cephalin gave about 60% of bromine, a few were as low as 56%. Probably the best way to explain low values in the petroleum-ether-insoluble fraction is by reasoning that any of the partially oxidized fatty acids, when brominated and separated as above described, would go into this fraction. It is extremely difficult to be sure that the product has been freed from such oxybromo acids. They would lower the bromine value. It is almost impossible, even with the precautions adopted against oxidation in this investigation, to say with certainty that no unsaturated bonds in the fatty acids had been saturated with oxygen. The principle evidence of oxidation in the cephalin was a darkening in color. Cephalin (4) and (8) were about white; a faint yellowish color was evident when a compact mass was observed. The amount of oxidation was very small, at most.

It is probably not a hexabromobehenic or lignoceric acid, because one would expect these derivatives to have melting points about 180° whereas the best samples of cephalinic bromide melted at about 120°. The bromides of the higher acids would undoubtedly be even less soluble in ether than hexabromostearic acid, but the bromide of cephalinic acid is soluble in ether, though not so readily soluble as the tetrabromostearic acid. Besides, the melting point is too high for the tetrabromo compound. There seems to be no tendency, even at high temperatures, to form the soluble linolic bromide. The appearance of cephalinic bromide is also different from linolic bromide. The former crystallizes out of ether, slowly but continuously as the ether evaporates, in rather heavy plates, while the latter does not crystallize till the ether solution is concentrated, and then it separates as needles or as a semisolid mass containing the liquid as well as solid tetrabromide. The characteristics of the cephalinic bromide agree best with Thudicum's[1] data on the barium salt. From his results cephalinic acid would be probably an hydroxypalmitic or stearic acid with four unsaturated bonds in it. The data in this paper agree fairly well with a tetrabromo hydroxypalmitic acid.

Clupanodonic Acid.

The ether-insoluble bromine fraction gave bromine values ranging from 67% to 69.4%. Several analyses on the best cephalin preparations follow:

	Br.
Beef cephalin (4) 0.0827 g. bromine compound	67.7%
Sheep cephalin (4) 0.2144 g. bromine compound	69.4%
Sheep cephalin (8) 0.1967 g. bromine compound	68.8%
Sheep cephalin (4) 0.2260 g. bromine compound	68.7%

The theoretical percentage of bromine in octobromostearic acid is 69.6. The above values agree as well as one could expect, considering the possibilities of slight oxidation during the preparation and hydrolysis of the cephalin. However, the difference in amount of bromine in $C_{18}H_{28}O_2Br_8$ and $C_{20}H_{32}O_2Br_8$ is but 2%. There is some evidence for believing that the latter compound is one of the bromination products of liver lecithin. It is entirely possible, then, that the ether-insoluble bromo compound is not a derivative of clupanodonic acid but of the next higher homolog. The values more nearly agree with clupanodonic acid, however.

This difficulty was not solved by melting-point determinations on the bromo compound. When heated rather rapidly it darkens at 220° but does not melt. Because of the uncertainty of this darkening point it is not a reliable way of differentiating between the two homologs. Probably both darken between 210° and 220° when the temperature is rapidly raised to that point.

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[1] Thudicum, "Die chemische Constitution des Gehirns," p. 149 (1901).

General Statements.—From the data presented it is reasonably certain that the two fatty acids in largest amounts in cephalin are stearic and oleic.

The evidence for clupanodonic acid is rather conclusive though not entirely so. Other methods besides the bromination one are being tried to clear up this point.

The greatest uncertainty centers around cephalinic acid. The solubilities and appearance of the bromo derivative strongly suggest linolic acid, but the bromine value is too high. The melting point and percentage of bromine indicate an impure linolenic acid or a higher homolog. Several lines of attack are being carried on the more clearly to understand this acid.

Summary.

1. Somewhat more than one-fourth of the acids of cephalin is in the form of stearic, more than one-half oleic, one-tenth cephalinic, and one-twentieth clupanodonic.

2. From the varying amount of these acids present it is concluded that they are present in mixed cephalins, these various cephalins being closely associated.

3. There is no marked difference, either, in the kind or amount of acids in sheep and beef brain cephalins.

4. The evidence presented strongly indicates ordinary stearic and oleic acids as the principal fatty acids of cephalin.

5. The evidence for the identity of clupanodonic and cephalinic acids is not conclusive.

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THE ACTION OF ANHYDROUS ALUMINIUM CHLORIDE UPON UNSATURATED COMPOUNDS.

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Received May 10, 1916.

It is known that the product obtained by cracking petroleums with aluminium chloride under certain conditions consists very largely of saturated hydrocarbons; whereas distillation under pressure results in a large percentage of unsaturated compounds. One possible explanation of this peculiarity is that the aluminium chloride combines with any unsaturated hydrocarbons formed in the reaction and holds them back from the distillate. The simple experiment of shaking ordinary gasoline with the anhydrous chloride showed that by this treatment all of the unsaturated hydrocarbons are converted into a dark-colored sludge, and that the gasoline resulting gives no color with concentrated sulfuric acid. Petroleum ether acts in a similar way.

A careful review of the literature discloses the fact that very little work