LXI. A COMPARISON BETWEEN THE MOLECULAR WEIGHTS OF PROTAGON AND OF THE PHOSPHATIDE AND CEREBROSIDES OBTAINABLE FROM IT.

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INTRODUCTION.

The following observations were carried out with the object of obtaining fresh and conclusive information concerning the question whether protagon is a mixture of phosphatides and cerebroside or whether it contains these substances in chemical combination. All authors are agreed that it is possible to prepare from protagon two cerebroside, namely cerebrin and homocerebrin (phrenosin and kersin of Thudichum), and a phosphatide, sphingomyelin. But while according to Thudichum and his followers protagon is simply a mixture of these three substances, together perhaps with a number of other simpler substances, the followers of Gamgee maintain that protagon contains these substances in chemical combination which is easily broken up with the liberation of the constituent phosphatide and cerebroside, just as haemoglobin is easily broken up into globin and haematin.

Hitherto all attempts to decide this question have been made by studying the behaviour of protagon on the one hand and of cerebrin, homocerebrin and sphingomyelin and mixtures of the three on the other hand towards certain solvents such as alcohol, chloroform, pyridine and others. But this line of argument has not only failed to bring about an agreement but has actually led to an impasse.

The observations of one group of authors are incompatible with those of another. Thus Thudichum and Rosenheim and Tebb state that protagon has

an indefinite and variable melting point, contains relatively large amounts of potassium, and completely alters its composition on recrystallisation from large volumes of alcohol; in other words, its behaviour is that of a mixture of a phosphatide and cerebrosides. On the other hand, on subjecting these statements to re-examination Gamgee, Roscoe, Baumstark, Ruppel, and Cramer find that protagon has a definite melting point, is free from potassium and retains its composition on recrystallisation from large and small volumes of alcohol; in other words its behaviour, according to these authors, is not that of a mixture of a phosphatide and cerebrosides. There is therefore hardly any common ground on which a discussion as to the nature of the substance in question is possible along these lines. Indeed the only conclusion an impartial observer can draw, is that the substance investigated by Thudichum and his followers is not identical with the one studied by Gamgee and his followers. Nor are these contradictions to be found only between the statements of those who attack and those who defend the existence of protagon as a definite compound. The observations of Posner and Gies that protagon retains its composition when dissolved in warm alcohol and cooled immediately, however large the volume of alcohol, is diametrically opposed to the statements of Rosenheim and Tebb. No explanation has ever been offered by the latter observers to account for this contradiction, and it is difficult to understand how these authors can quote each other in support of their views.

A new and more decisive line of argument is necessary in order to settle this question. It is offered in this paper and is as follows. The chemical composition of cerebrin and homocerebrin is fairly well known owing mainly to the work of Thudichum, Kossel and Freytag, Thierfelder and Levene and Jacobs. Thierfelder [Kitagawa and Thierfelder, 1906] from his observations on the products of acid hydrolysis of cerebrin, which is either identical with or closely allied to cerebrin, has calculated the formula $C_{46}H_{93}O_9N$ which has also been accepted by Levene and Jacobs [1912] as the result of their analysis. The molecular weight of cerebrin according to this formula would therefore be 827. In the case of the closely allied homocerebrin the molecular weight has been determined directly by means of the elevation of the boiling point by Kossel and Freytag and was found to lie between 945 and 1027. In the case of sphingomyelin our knowledge depends mainly on the statement of Thudichum, who from a study of the products of hydrolysis gives to it the formula $C_{52}H_{104}O_9N_2P$. The molecular weight of sphingomyelin would be, accordingly, 931.

The three substances which can be obtained from protagon therefore have molecular weights of such an order of magnitude that, if they follow Raoult's law, a definite elevation of the boiling point should be noticeable in solutions of moderate concentrations (3 %-5 %) of these substances, especially if one uses a solvent with a relatively high constant such as chloroform. One gram of a substance with the molecular weight 1000 would, in a 4 % solution in chloroform, elevate the boiling point by about 0.10° . If therefore protagon were a mixture of these substances it should, with the concentrations given, produce a distinct elevation of the boiling point of chloroform.

On the other hand, if protagon contained these substances in chemical combination it would have a molecular weight at least approximating to 3000. It may, of course, be considerably higher; in fact Cramer calculated from the amount of sulphur in protagon a molecular weight of 5778. If therefore protagon were a combination and not a mixture of cerebrosides and phosphatides the boiling point of a 3 % to 5 % chloroform solution of protagon should show either no elevation at all of the boiling point of chloroform or only a very slight one compared with that produced by cerebrin, homocerebrin, sphingomyelin or mixtures of these substances.

Observations on the boiling point of chloroform solutions of these substances by means of Beckmann's method will therefore give a final and decisive answer to the question whether protagon is a chemical combination or a mixture of phosphatides and cerebrosides. And since with substances of high molecular weights the determination of the molecular weights can only be approximate, it may be pointed out that this answer is not dependent on the exact numerical evaluation of the molecular weights nor upon slight quantitative differences between them, but upon differences of such an order of magnitude as to be qualitative.

EXPERIMENTAL.

The observations were made by Beckmann's method with an apparatus having an electrical heating device. The amount of chloroform used was measured in every case by volume, not by weight, and amounted to 25 cc. The chloroform was "chloroform from chloral" except in one case when "chloroform from acetone" was used. In almost every case a reading of the boiling point of the pure solvent was taken both before and after the experiment in order to be able to correct for changes due to variations in

barometric pressure. The boiling point of a solution was taken as the point where three successive readings at intervals of five minutes gave constant results.

Protagon was prepared from ox brain by the method of Wilson and Cramer: after completely exhausting the fresh minced brain tissue by repeated extraction with cold acetone and cold ether at room temperature the residue was extracted rapidly with boiling alcohol. Two alcohol extracts only were made. The white precipitate, obtained on cooling the alcoholic extract, was twice recrystallised. No more than ten ox brains were worked up at any one time. The process was repeated with new quantities of fresh ox brains until about 12 g. of dry protagon had been obtained in the form of a snow-white pulverulent powder. It is not advisable to work with large quantities of brain tissue.

Part of the protagon obtained in this way was used for the preparation of cerebrin and homocerebrin, by boiling it with baryta water. This method, which was used originally by Parcus and by Kossel and Freytag, has been re-introduced again recently by Lorrain Smith and Mair [1910] and by Loening and Thierfelder [1911]. Protagon is made into a fine emulsion with saturated baryta water, heated under a reflux condenser in a vigorously boiling water bath for one hour and the mixture filtered. The residue, after boiling with a mixture of alcohol and acetone, is repeatedly extracted with boiling acetone from which on cooling a mixture of cerebrin and homocerebrin separates out. The mixture thus obtained was recrystallised twice from boiling acetone and was obtained eventually in the form of a white powder more granular in appearance than protagon.

Since it was the object of these observations to compare the behaviour of protagon with that of a mixture of the cerebrosides which can be prepared from it, the separation of the two cerebrosides was not carried out. The observations made with protagon, and with the mixture of cerebrin and homocerebrin respectively, are recorded in Tables I and II.

These observations show clearly that the size of the protagon molecule is very much larger than that of the molecules of cerebrin and homocerebrin. Indeed the protagon molecule is so large that even in concentration exceeding 5% it fails to produce an elevation of the boiling point of chloroform. Now it is possible to calculate from the amount of galactose liberated from protagon, cerebrin, and homocerebrin respectively that *one gram* of protagon contains 0.6 to 0.7 gram of cerebrosides and 0.4 to 0.5 gram of sphingomyelin. Even if one assumed that sphingomyelin had a very much larger molecule

than the data of Thudichum would indicate, or if one assumed that for some reason or other it did not follow Raoult's law, perhaps by aggregating in solution into a colloidal form,—even then the elevation of the boiling point produced by *one gram* of protagon ought to be at least as great as that produced by 0.6 to 0.7 gram of the cerebroside obtained from it. As a matter of fact, however, 0.7 gram of a mixture of cerebrin and homocerebrin

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TABLE I.

Boiling point of chloroform solutions of protagon.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	<i>e</i>	Barometer
I.	25	—	59.62		
		0.6664	59.62	0	734
		—	59.62		
II.	25	—	59.92		
		0.9730	59.92	0	742
		—	59.92		
III. ¹	25	—	60.50		
		0.9430	60.45	-0.05	not read
		1.5920	60.45	-0.05	
		—	60.50		

¹ In observation No. III chloroform from acetone was used.

TABLE II.

Boiling point of a chloroform solution of a mixture of cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	<i>e</i>	Barometer
IV.	25	—	59.50		
		0.7305	59.53	+0.03	
		1.5140	59.63	+0.13	728
		—	59.50		

is sufficient to produce a distinct elevation of the boiling point of 25 cc. of chloroform, while even double the quantity of protagon is incapable of producing that effect. It follows, then, that the observations recorded in Tables I and II are incompatible with the view that protagon is a mixture of sphingomyelin, cerebrin and homocerebrin.

It will be noted that the elevation of the boiling point produced by cerebrin and homocerebrin increases with increasing concentration out of proportion to the amounts of substance added. This irregularity has no

bearing on the problem under discussion but it is of some general interest since a similar phenomenon has been observed in the case of other substances with large molecules, for instance in the case of colloidal solutions of silicic acid, tungstic acid and molybdic acid. It will also be noted in one case (Experiment No. III) that the boiling point of a chloroform solution of protagon instead of remaining constant showed a slight diminution, which, however, was not increased on adding more protagon. Since in this case a less pure sample of chloroform ("chloroform from acetone") was used, the irregularity may probably be referred to this fact.

Although a chloroform solution of protagon shows no elevation of the boiling point, yet the presence of protagon does not hinder the boiling point of chloroform being affected by the addition of another substance. This is shown by the fact that the addition of a weighed quantity of naphthalene to 25 cc. of a chloroform solution containing 0.6664 gram of protagon produced an increase of the boiling point commensurate with its molecular weight, 128. Thus the addition of 0.4240 g. naphthalene gave an elevation of the boiling point $e = 0.37^\circ$, molecular weight calculated, 119. The further addition of 0.4306 g. naphthalene produced a further elevation, $e = 0.37^\circ$, molecular weight calculated, 121.

In order to compare the molecular size of sphingomyelin with that of cerebrin, homocerebrin, and protagon, sphingomyelin was prepared by heating crude protagon with pyridine to 50° for 20 minutes. The precipitate which falls out on cooling the pyridine is removed by filtration. After washing with cold alcohol and drying in vacuo it had a phosphorus percentage of 2.3 %; it represents according to Rosenheim and Tebb [1910] sphingomyelin with a slight admixture of cerebroside. The pyridine filtrate from sphingomyelin when poured into an excess of acetone gives a precipitate consisting of cerebroside with a slight admixture of sphingomyelin. The sphingomyelin obtained in this way was not freed from its slight admixture of cerebroside, since the object of these observations is to obtain comparative values for protagon on the one hand, and the substances or mixtures of the substances which can be obtained from it, on the other.

The observations made with sphingomyelin prepared in this way are given in Table III. It will be seen that sphingomyelin produces a distinct elevation of the boiling point of chloroform, thus indicating that the size of the molecule of sphingomyelin is of about the same order of magnitude as that of cerebrin and homocerebrin and very much smaller than that of protagon. The observations with sphingomyelin, therefore, confirm the

conclusions arrived at from the observations with cerebrin and homocerebrin.

The preparation of sphingomyelin and the cerebrosides from protagon by heating the latter with pyridine made it possible to compare protagon with a mixture of sphingomyelin and cerebrosides in approximately the

TABLE III.

Boiling point of chloroform solution of sphingomyelin (with slight admixture of cerebrosides).

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	<i>e</i>	Barometer
		—	60.56		
V.	25	0.6842	60.60	+0.04	754
		—	60.35		
VI.	25	1.0948	60.42	+0.07	752

TABLE IV.

Boiling point of a chloroform solution of a mixture of sphingomyelin, cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	<i>e</i>	Barometer
		—	60.35		752
		1.0948	60.42	+0.07	
		(sphingomyelin)			
		+		+	
		0.7210	60.45	+0.03	
		(cerebrosides)			
		+		+	
		0.7196	60.47	+0.02	
		(cerebrosides)			
		—	60.25		749

Note that in this table the changes in the elevation of the boiling point produced by the successive addition of sphingomyelin and cerebrosides are given separately with reference to each single addition and not collectively. For a detailed discussion see text.

same proportions in which they can be obtained from it. For that purpose the chloroform solution of sphingomyelin obtained in Experiment No. VI, Table III, was used. After having obtained a constant value for the boiling point of this solution the cerebrosides obtained by the pyridine treatment described above were added. The results are given in Table IV. There was a rapid fall of barometric pressure on the day on which this observation was carried out. Experiment No. V, Table III, was made on the forenoon of the

same day, Experiment No. VI, Table III, and the observations recorded in Table IV, on the afternoon of that day. It will be seen that the barometer fell from 754 mm. at 9 a.m. to 749 mm. at 3 p.m. There is, of course, a corresponding fall of the boiling point of the pure solvent. Since in the observations recorded in Table IV the boiling points of three different solutions were determined, one hour and a half elapsed between the beginning and the end of these observations, and even during that time a distinct fall in boiling point of the pure solvent was noticed.

Although the barometric variations would tend to diminish any elevation in the boiling point produced by the addition of cerebrosides, there is nevertheless clear evidence of a rise in the boiling point of the chloroform solutions of sphingomyelin on every addition of cerebrosides. Taking the last observations it will be seen that a mixture of about 1.1 gram sphingomyelin and 1.4 gram cerebrosides having a phosphorus percentage of 1 % corresponding to that of protagon produced a total rise of 0.22° . Of this rise 0.07 is due to the sphingomyelin present, and consequently a rise of 0.15 is due to the cerebrosides. This is approximately the same value as that obtained before in the observation recorded in Table II. In other words, the effect produced by a mixture of sphingomyelin, cerebrin and homocerebrin is simply the additive effect produced by the substances constituting the mixture. If protagon were a loose molecular compound of phosphatides and cerebrosides which could be reconstituted by simply mixing these substances together as has been suggested by some writers, the addition of cerebrosides to the solution of sphingomyelin should diminish the elevation of the boiling point produced by sphingomyelin. As a matter of fact the opposite takes place. The observations show then again that protagon is not a mixture of sphingomyelin, cerebrin and homocerebrin, but that it must contain these substances in chemical combination. They also show that it is not possible to reconstitute protagon by mixing together in certain proportions the cerebrosides and phosphatides which can be prepared from it.

DISCUSSION.

The observations recorded in this paper show that the molecule of protagon is very much larger than that of cerebrin, homocerebrin or sphingomyelin. It follows therefore that protagon is not a mixture of these substances but holds them in chemical combination. It is also shown that the behaviour of a mixture of cerebrin, homocerebrin and sphingomyelin is

different from that of protagon and that it is not possible to reconstitute protagon by simply mixing together these simpler substances in certain proportions. This again shows that protagon is not a mixture of phosphatide and cerebroside. It also shows that protagon is not a loose molecular compound of these substances which is formed when these substances occur together in solution.

While the observations recorded in this paper establish the fact that protagon is a chemical combination and not a mixture of cerebroside and phosphatides, they do not answer the entirely different and much less important question whether protagon is only one such combination of phosphatides and cerebroside or a mixture of several such combinations.

Although the essential difference between these two questions is obvious, it is necessary to emphasise it, because they have been confused in the past. Those who held with Gamgee that protagon is not a mixture of phosphatides and cerebroside have been represented by their opponents as maintaining that protagon is not a mixture; they have been criticised for not bringing forward any evidence to prove that protagon is a single substance, and from their inability to do so it has been argued, not that protagon is a mixture, which might be logical, but that protagon is a mixture of phosphatides and cerebroside, which is quite illogical. In this way the entire protagon question has become obscured and distorted. It is obvious that the fact that protagon is a chemical combination of cerebroside and phosphatides does not exclude the possibility that protagon consists of a mixture of several such combinations. This possibility has in fact been pointed out by Kossel and Freytag and by Cramer. The question whether any complex substance isolated from tissues is a single substance or a mixture of homologous substances is as difficult to decide in the case of protagon as in the case of haemoglobin or any other protein. Even in the case of many phosphatides and cerebroside it is not yet settled. Fortunately no considerations of great theoretical or practical importance depend on the question whether protagon is a single substance or a mixture of several protagon. But considerations of quite a different order of importance are involved in the question whether protagon is a mixture or a chemical combination of cerebroside and phosphatides. This, however, does not appear to be clearly understood if one may judge from the statements frequently made in the literature, so that a brief indication of the significance of this question is called for.

If the views of Thudichum were correct, phosphatides and cerebroside

must be considered to be the most complex forms of lipoids present in tissues. Many text-books actually represent the chemistry of lipoids in this way. Now phosphatides are lipoids containing phosphorus but no galactose; cerebrosides are substances containing galactose but no phosphorus. Any substance isolated from tissues which contains both phosphorus and galactose is, from this point of view, considered to be a mixture of phosphatides and cerebrosides and is, therefore, subjected to methods of purification, no matter how drastic, until substances either free from phosphorus or free from galactose are obtained. The substances obtained after such treatment are then considered to exist preformed in the tissues.

We find accordingly substances obtained by boiling brain with baryta water unhesitatingly accepted as preformed constituents of nervous tissue. But since it has been shown in this paper that it is possible to isolate from nervous tissue a substance having a larger molecule than either phosphatides or cerebrosides, and since this substance contains both galactose and phosphorus in its molecule, it is necessary to recognise the existence of a group of lipoids, more complex than either phosphatides or cerebrosides. Since in the case of protagon, which is the best known representative of this group, cerebrosides preponderate in the building up of the molecule, and since protagon in its physical properties resembles cerebrosides and is unlike the typical phosphatides, the term "phosphorised cerebrosides" or briefly "phospho-cerebrosides" as proposed by Cramer [1911] seems most convenient.

The observations recorded in this paper also furnish an answer to the much debated question whether protagon can be decomposed by the application of solvents such as alcohol and pyridine. As pointed out in the introduction, up to the present time the protagon controversy has centred entirely round this question. It has been shown that by the long repeated application of warm alcohol it is possible to prepare from protagon cerebrosides and phosphatides. The fact itself is not disputed; what is in dispute is the interpretation which is to be placed upon it. Thudichum and his followers deny that this process takes place under conditions involving the decomposition of protagon and they describe this process as a "fractional crystallisation." But their statements in regard to this point are always made dogmatically and no evidence is offered in support of them. Gamgee and his followers claim that protagon is easily decomposed by alcohol and Wilson and Cramer have shown that the conditions which make a separation of protagon into phosphatides and cerebrosides possible also produce a distinct change in the physical constants of protagon. More recently Rosenheim and Tebb have

used pyridine as a means of preparing the cerebrosides and sphingomyelin from protagon. Rosenheim [1913] describes the method as a separation by cold pyridine although the process involves a preliminary heating to 40° and claims again that pyridine is an inert solvent and that the process excludes the possibility of decomposition. Again no evidence is offered in support of this claim and it is clear that, if the possibility of a decomposition of protagon by alcohol is admitted, as it is now admitted by Rosenheim, a solvent like pyridine which has a strongly alkaline reaction is even more likely to bring about such a change.

The method of separation by pyridine has been used and described in this paper. The molecular weights of the substances obtained by this method have been examined both when isolated and when mixed together again in the proportions in which they were obtained from protagon. Since protagon has a very much larger molecule than the substances or mixtures of substances which can be obtained from it by the use of pyridine or alcohol, it is evident that the methods used for the preparation of cerebrosides and sphingomyelin from protagon involve a decomposition of protagon.

SUMMARY.

Observations have been made by Beckmann's method on the elevation of the boiling point of chloroform produced by the addition of protagon on the one hand, and of the phosphatide (sphingomyelin) and the cerebrosides (cerebrin and homocerebrin) obtainable from protagon, on the other hand. The observations show that protagon has a very much larger molecule than the phosphatide and cerebrosides mentioned, either when separated or when mixed together. It follows that protagon is neither a mixture of these substances, nor a loose molecular compound formed when sphingomyelin, cerebrin and homocerebrin occur together in solution. The observations prove that protagon is a chemical combination of cerebrosides and a phosphorus-containing lipoid (sphingomyelin). It represents a group of lipoids more complex than either phosphatides or cerebrosides which is most suitably classified by the term "phospho-cerebrosides."

While these observations prove that protagon is a "phospho-cerebroside," *i.e.* a chemical combination of a phosphatide and cerebrosides and not a mixture of these substances, they afford no evidence with regard to the entirely different problem whether protagon is a simple "phospho-cerebroside" or a mixture of several "phospho-cerebrosides."

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References to the older literature are not given in this paper, as a complete reference to the literature on protagon and cerebrosides up to 1910 is given in the chapter by Cramer on Protagon, Cerebrosides and allied substances in the *Biochem. Handlexicon*.