ON THE PHOSPHORUS PERCENTAGE OF VARIOUS SAMPLES OF PROTAGON

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In the most recent contribution to the protagon question Posner and Gies¹ have on the basis of a careful experimental investigation confirmed their conclusion that protagon is not a well-defined chemical substance, but a mixture of various substances, differing widely in the amount of phosphorus they contain and in their solubility in alcohol and ether. Although the results on which they base their view are supported by a wealth of analytical data, the interpretation which they put upon these results is open to criticism. A more detailed discussion of this subject must be reserved for a future occasion.

In this communication we intend to bring forward some positive evidence in favour of the view that protagon is an individual substance and not a mixture. It must be pointed out, however, that a final and decisive proof of the homogeneous nature of a substance, even of a less complex nature than protagon cannot be brought until the substance in question can be synthesized. In default of this the constancy of its physical and chemical properties is generally considered to be sufficiently trustworthy evidence. In order to definitely deprive a substance like protagon, possessing characteristic physical and chemical properties, of its existence as a chemical individual, it is necessary to isolate and identify the various substances constituting the mixture—a task which in view of the difference in the physical and chemical properties of these substances should be a comparatively easy one.

1. Posner and Gies, *Journal of Biological Chemistry*, Vol. I, p. 59, 1905.

Wörner and Thierfelder¹ have indeed claimed to have isolated a phosphorus-free substance from protagon which they called cerebron. In a former paper² the similarity of this substance with Gamgee's pseudocerebrin was pointed out.

The conclusion that these two substances were identical was confirmed by a letter from Professor Gamgee containing some valuable new data, which was received a few weeks after the publication of the paper referred to. With his permission we give here the paragraph referring to this subject. 'When Thierfelder's paper on cerebron appeared, I at once identified his body as my pseudocerebrin. The absolute identity of my body with his is proved not merely by the concordance of the ultimate organic analysis made by him and by me but by the result of the determination of the melting point. Unfortunately I did not publish these, but on referring to my laboratory note-book of this period, I find bearing the date July 11th, 1879, as a result of observations on the melting point of pseudocerebrin (in capillary tubes),

Becomes slightly orange at 197° C.

Melts at 210° C.

'Wörner and Thierfelder obtained identical results. They say, "Erst bei 200° wird es leicht gelblich. Bei langsamen Erhitzen bei 209°, bei schnellem bei 212° schmilzt es zu einer klaren gelblichen Flüssigkeit." No closer concordance between melting point determinations could be obtained and it is obvious not only that we have worked on the same body, but that, although I made no pretensions in reference to the purity of pseudocerebrin, the body I analysed was identical with and as pure as Thierfelder's.'

In a recent paper Thierfelder³ has frankly acknowledged the identity of cerebron and pseudocerebrin.

According to Gamgee⁴ pseudocerebrin exists together with protagon and is extracted together with protagon from the brain.

1. Wörner und Thierfelder, *Zeitschrift für physiolog. Chemie*, Vol. XXX, p. 542, 1900.

2. Cramer, *Journal of Physiology*, Vol. XXXI, p. 31, 1904.

3. Thierfelder, *Zeitschrift für physiolog. Chemie*, Vol. XLIII, p. 21, 1904.

4. Gamgee. *Text-book of Physiological Chemistry*, Vol. I, London, p. 44, 1880.

Being less soluble in alcohol than protagon, Gamgee was able to separate it from protagon by simple recrystallisation. If his view is correct, it is possible to remove pseudocerebrin entirely from the phosphorised matter, which can be obtained as a definite chemical compound of a constant phosphorus percentage. According to Posner and Gies pseudocerebrin is one of the substances of which the mixture called protagon is made up. Every sample of protagon that has ever been prepared, even Gamgee's protagon, contains, according to them, considerable quantities of pseudocerebrin. If their view is correct, the phosphorus percentage of samples obtained in repeated crystallisations or by different methods of preparation should not remain constant. Gamgee,¹ however, found that after repeated recrystallisation the phosphorus content of the protagon remained constant. Posner and Gies have recrystallised protagon as often as ten times. They found the phosphorus percentage of the crystals separating out (0.93 per cent.) and of the substance in the mother liquor (1.02 per cent.) to be almost identical²—surely the most conclusive proof of the homogeneous nature of the substance they were dealing with. The significance of this result does not seem to have been recognised by the authors. According to them even this sample contained considerable quantities of pseudocerebrin as evidenced by the effect of their method of fractionation.

There is further the evidence³ that by using a method quite different from that of previous observers and entailing the use of relatively high temperatures, a substance was extracted from brain which was identical with the protagon of previous workers. If we accept the view of Gies and his collaborators and consider protagon to be a mixture of substances varying in their solubility and in their phosphorus percentage, we should expect to obtain different mixtures by extracting brain tissue by different methods, especially by using different solvents.

In order to apply solvents such as chloroform to brain tissue it

1. Gamgee, *Text-book of Physiological Chemistry*, p. 428.

2. Posner and Gies, *loc. cit.*, p. 107.

3. Cramer, *loc. cit.*

is necessary to remove the water. This we have done by following the method employed by Bünz¹ for the preparation of cholesterin. Fresh ox brains, after having been passed through a mincing machine, were rubbed up in a mortar with anhydrous sodium sulphate, until a dry powder was formed. This powder was extracted with cold ether until all the lecithin and cholesterin had been removed. The residue was used for the preparation of protagon by adding the various solvents and extracting with the boiling solvent for one to two minutes. The phosphorus percentage of the various samples was estimated by fusing with soda saltpetre mixture and weighing the phosphoric acid as magnesium pyrophosphate.

Protagon A.—The dry powder was extracted with 1500 c.c. of boiling absolute alcohol. The crystals separating out were recrystallised three times, the second and third recrystallisation were submitted to analysis.

Second recrystallisation 1·34 per cent. P.

Third recrystallisation 1·07 per cent. P.

Protagon B.—A second sample of protagon was prepared in exactly the same way. The second recrystallisation gave on analysis—

Second recrystallisation 1·25 per cent. P.

One portion of this sample was recrystallised out of boiling absolute alcohol.

Third recrystallisation 1·05 per cent. P.

Protagon C.—Another portion was recrystallised out of boiling glacial acetic acid and contained 0·96 per cent. P.

Protagon D.—The dry powder was extracted with 1500 c.c. of boiling methylalcohol. The substance separating out on cooling was recrystallised three times out of boiling methylalcohol. The analysis of these samples gave the following figures :—

Second recrystallisation 0·94 per cent. P.

Third recrystallisation 0·97 per cent. P.

Protagon E.—A sample of protagon was prepared in the manner described for samples A and B. After one recrystallisation it contained 1·22 per cent. P. It was recrystallised once more out of methylalcohol

1. Bünz, *Zeitschrift für physiolog. Chemie*, Vol. XLVI, p. 47, 1905.

in the following manner : a small quantity of the solvent, which was not sufficient to dissolve all the protagon, was added first and decanted after having kept the solution boiling for one minute. The same process was repeated with another small quantity of methylalcohol. The crystals separating out in these two fractions gave on analysis—

First Fraction 1·14 per cent. P.

Second Fraction 1·05 per cent. P.

Protagon F.—The dry powder was extracted with 500 c.c. of boiling chloroform. After cooling 1500 c.c. of ether were added. A white precipitate formed, which was analysed without further purification, as only small quantities could be obtained in this way. It contained 1·18 per cent. P.

On cooling the chloroform extract nothing separated out. After evaporating the bulk of the chloroform in vacuo, a viscous semi-fluid mass remained, which did not crystallise on standing.

None of the methods can be recommended for practical use in the preparation of protagon. Of all the solvents used absolute alcohol gave the best results. Nor does the method of removing the water by mixing the brain pulp with anhydrous sodium sulphate offer any advantage as it increases too much the bulk of the matter to be extracted. A better way of removing the water is to treat the brain pulp with cold alcohol and ether. In this way a dry powder is obtained and at the same time superfluous matter (lecithin, cholesterolin, etc.) is removed, so that the bulk of the matter is greatly reduced. Details of this method will be given in a later paper.

The results of our analyses can be seen more clearly from the following table.

Solvent ...	...	...	...	Aethyl-Alcohol		Glacial Acetic	Methyl-Alcohol		Chloroform
Sample of Protagon ...	...	...	...	A	B	C	D	E	F
Crude crystalline product	...	...	...	—	—	—	—	—	1·18
First recrystallisation	...	...	...	—	—	—	—	—	—
Second recrystallisation	...	...	...	1·34	1·25	—	0·94	{ 1·14 1·05	—
Third recrystallisation	...	...	...	1·07	1·05	0·96	0·97	—	—

It will be seen that the phosphorus percentage of these various samples of protagon, extracted by different solvents, is remarkably constant. Comparing only the most highly purified products, A, B, C and D, the greatest difference is = 0.11 between sample A and sample C. Sample F, prepared by precipitation with ether, is of special interest as, according to Gies,¹ protagon contains a considerable quantity of an ether soluble substance, poor in phosphorus. If this is correct, the method employed for Sample F should yield a substance richer in phosphorus than the other samples of protagon. This was not found to be the case.

A sample of protagon which was prepared by the coagulation method (heating the brain emulsion with a concentrated sodium sulphate solution in the boiling water bath and extracting the coagulum with boiling absolute alcohol) contained after three recrystallisations 1.07 per cent. P.

The figures which have been obtained by previous workers may be given here for comparison.

Liebreich ¹	...	...	...	...	1.23 per cent. P.
Gamgee ²	...	...	...	...	1.07 per cent. P.
Baumstark ³	...	...	...	...	1.02 per cent. P.
Kossel and Freitag ⁴	...	...	...	...	0.97 per cent. P.
Ruppel ⁵	...	...	...	...	1.13 per cent. P.
Cramer ⁶	...	...	...	...	1.07 per cent. P.
Posner and Gies ⁷ (Gamgee's method)					0.93 per cent. P.
(Cramer's method)					0.93 per. cent P.

1. Liebreich. *Liebig's Annalen*, Vol. CXXXIV, p. 29, 1864.
2. Gamgee. *Text-book of Physiological Chemistry*, p. 428.
3. Baumstark. *Zeitschrift für physiolog. Chemie*, Vol. IX, p. 145, 1888.
4. Kossel and Freitag. *Ibid.*, Vol. XVII, p. 431, 1892.
5. Ruppel. *Zeitschrift für Biologie*, Vol. XXXI, p. 86, 1892.
6. Cramer. *Loc. cit.*
7. Posner and Gies. *Loc. cit.*, pp. 97, 109.

CONCLUSIONS

The close agreement between the phosphorus percentage of various samples of protagon prepared by the most diverse methods is strong evidence in favour of the view that protagon is an individual substance of a well-defined chemical composition. Even more conclusive evidence is afforded by the observation of Posner and Gies, that after ten times repeated crystallisation the protagon crystals separating out have the same phosphorus percentage as the substance in the mother liquor.

The view that protagon is a mixture of substances differing in their solubility and in their phosphorus contents is not compatible with these results and cannot be accepted until the substances constituting the mixture have been isolated.

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