THE PROTAMINE SALTS OF PHOSPHATIDES, WITH REMARKS ON THE PROBLEM OF LIPOPROTEINS*

By ERWIN CHARGAFF

(From the Departments of Biological Chemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York)

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In the course of a study of the rôle of protamines in blood coagulation, to be described in the paper immediately following (1), it was observed that a stable emulsion of highly purified cephalin gave a characteristic precipitate on addition of a solution of the protamine salmine. In contrast, neither lecithin nor sphingomyelin produced precipitates under similar conditions. This reaction which seemed to offer a convenient model for the formation of complexes between phosphatides and proteins warranted a closer investigation. That the precipitation of cephalin was not a simple flocculation due to a salt effect could be immediately seen from the fact that the isolated precipitates could not be reemulsified in water. On drying, powders of high nitrogen content were obtained (P:N = 1:4 or 1:5) which in contact with water swelled to form a rubber-like elastic mass. The products were soluble in organic solvents, could be recrystallized unaltered from ethyl acetate, and did not change their composition following treatment of their solutions in ether with dilute acids or reprecipitation with acetone.

From the behavior of the cephalin-salmine compounds it may be concluded that they are not simple adsorbates, but water-insoluble salts between the acidic cephalin and the strongly basic salmine. A study of the influence exerted by buffers of varying pH on this reaction showed that the immediate formation of the insoluble precipitate took place over the entire range examined; viz., from pH 2 to 11. This is noteworthy because of the possible

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conclusion that certain proteins not as markedly basic as salmine, the isoelectric point of which has been found at 12 (2), may form compounds with cephalin at a physiological pH.

With lecithin no such compound formation took place within the physiological range. In strongly alkaline buffers, however, at pH 10 and 11, there occurred the formation of insoluble compounds.

This significant difference in the behavior of cephalin and lecithin towards the protamine may be understood from a consideration of the polar characteristics of these phosphatides.

\[
\begin{align*}
\begin{array}{c}
\text{CH}_2\text{OR} \\
\text{CH}_2\text{OR} \\
\text{CH}_2\text{O} \\
\text{P} \\
\text{O} \\
\text{O}
\end{array}
\text{Lecithin} \\
\begin{array}{c}
\text{CH}_2\text{OR} \\
\text{CH}_2\text{OR} \\
\text{CH}_2\text{O} \\
\text{P} \\
\text{O} \\
\text{O}
\end{array}
\text{Cephalin}
\end{align*}
\]

Lecithin, which contains the strong base choline, is an internally neutralized compound. In recent determinations (3) its isoelectric point was found at 6.7. Cephalin, on the other hand, which contains the weaker base ethanolamine, has markedly acidic properties. According to Grün and Limpacher (4) and Rudy and Page (5) cephalin under certain conditions is titrated as a monobasic acid towards phenolphthalein, whereas lecithin is neutral. In a more recent investigation Fischgold and Chain (6) come to the conclusion that at acid reaction both lecithin and cephalin are able to bind 1 equivalent of $H^+$ ions, whereas at alkaline reaction only cephalin gives up 1 equivalent of $H^+$ ions.

As to the chemical nature of the extremely stable products obtained by the reaction of cephalin and salmine, the most likely assumption is that of salt formation between the strongly basic protamine and the acidic phosphatide. On the basis of their phosphorus and nitrogen contents the substances consisted of about 80 per cent of cephalin and of 20 per cent of salmine.

The bearing of the experiments here discussed on the problem of lipoproteins is obvious. Although the existence of lipid-pro-
tein complexes in nature has long been assumed, experimental work with regard to the nature of these complexes has been scanty. One group of workers chose the preparatory approach to the problem by attempting the synthesis of complexes between serum or egg albumin (8–11), zein (10), or caseinogen (12) and lecithin. That these attempts did not lead to stable compounds may, in the light of the results here reported, be partly due to the fact that lecithin, in contrast to cephalin, is not appropriate for model experiments of this nature. Another group of workers (13–15) investigated the influence of proteins on the flocculation of lecithin sols. In these studies cephalin does not seem to have been used either.

Because of the inconclusive results obtained by other workers, orienting experiments were carried out on the formation of compounds between highly purified samples of egg albumin and lecithin or cephalin at various pH levels. It was of great interest to find that also with egg albumin immediate compound formation appeared to occur only in the case of cephalin. The precipitation of cephalin complexes took place at pH 2, 3, and 4. The much narrower range within which cephalin-albumin complexes are formed, as compared with the formation of compounds with salmine, is in entire harmony with the position of the isoelectric points of the two proteins, egg albumin having its isoelectric point at 4.8, salmine at 12.2.

Lipoproteins have an important function in many biological processes. The natural activator of blood coagulation, the thromboplastic factor, apparently is a complex between cephalin

1 The indiscriminate use of the term lipoproteins (e.g. (7)) for complexes between proteins and phosphatides, sterols, glycerides, and fatty acids respectively is confusing. Besides, one ought to distinguish between compounds, as the phosphatides, containing functional groups which make possible a combination with proteins by primary valence forces, and the sterols, for example, which, if they combine with proteins at all, could only do so by an attraction due to secondary valence forces.

2 It will be of interest to study the formation of compounds between cephalin and proteins having more basic isoelectric points than egg albumin, which ought to take place within the physiological pH range. In view of the importance of cephalin-protein complexes in blood coagulation it should be noted that the isolation of two basic fibrinogen fractions has been reported with isoelectric points at pH 8.5 and 12.4 respectively (16) (compare, however (17)).
and a probably specific protein (18). The importance of lipid-protein complexes in immunology (19–21) can only be mentioned here.

When the salt-like nature of lipoproteins is put forward as a possible formulation of these compounds, it should be kept in mind that the fact that lecithin and cephalin usually occur together by no means connotes a common physiological function of the two. It is entirely possible that cephalin will be found to be a structural component of the organism, whereas lecithin may be concerned with the intermediary fatty acid metabolism. It is hoped that the use of the radioactive phosphorus isotope \( ^{32}P \) as a label will be of value for investigations of this type.

**EXPERIMENTAL**

**Material**

*Cephalin* — Two different samples of cephalin were used in these experiments. One was a freshly isolated preparation from beef brain, obtained by extraction of the acetone-dried organ with petroleum ether (b.p. 30–60°). This material\(^3\) was freed of cerebrosides by repeatedly freezing its solutions in ether and petroleum ether, and separated from lecithin by numerous precipitations with alcohol from petroleum ether solution. The cephalin, Fraction C-1, formed an almost white powder. Analysis, found, C 61.2, H 9.4, P 3.5, N 1.7, amino N 1.4, P:N = 1:1.1, P:amino N = 1:0.9.

The other cephalin preparation used was an older sample that had been extracted from sheep brain by means of ether and stored under \( \text{CO}_2 \) for some time. In order to purify this material two methods were attempted which, since they may be of general interest, will be briefly described. One method involved the chromatographic adsorption of the cephalin to aluminum oxide (activated according to Brockmann). A solution of 1.12 gm. of cephalin in 50 cc. of a mixture of equal parts of petroleum ether (b.p. 30–60°) and ligroin (b.p. 70–90°) was slowly filtered through an adsorption column (150 X 15 mm.). The chromatogram then was developed with 70 cc. of the petroleum ether-ligroin

\(^3\) We are indebted to Mr. H. D. Hoberman for this preparation.
mixture. The colorless filtrate was concentrated in vacuo, and the ether solution of the residue was centrifuged, concentrated, and precipitated with acetone. The cephalin, Fraction C-2, weighed 0.44 gm. and formed a white powder. Analysis, found, C 55.8, H 9.0, P 4.0, N 1.9, amino N 1.7, P:N = 1:1.

The second method consisted in the distribution of the material between two solvents; viz., petroleum ether-ligroin and diacetone alcohol, CH₃·CO·CH₂·C(CH₃)₂OH. While the dry solvents are perfectly miscible, the addition of 1 per cent of water to the diacetone alcohol is sufficient to produce a sharp separation into two layers. Of the crude cephalin, 10.0 gm. were dissolved in 100 cc. of petroleum ether-ligroin (1:1), 40 cc. of freshly distilled diacetone were added, followed, after being mixed, by 0.4 cc. of water. The lower layer removed a considerable amount of impurities. The process was repeated five times, whereupon the solution of the cephalin in petroleum ether-ligroin was slowly dropped into 300 cc. of chilled absolute alcohol. The cephalin, Fraction C-3, weighed 7.4 gm. and formed a faintly yellow powder. Analysis, found, C 54.3, H 8.6, P 4.2, N 1.8, amino N 1.6, P:N = 1:0.94.

Lecithin—The lecithin used was freshly prepared from beef brain, as described above for the cephalin Fraction C-1. For purification it was converted into the cadmium chloride double salt. This salt was washed with ether, recrystallized from ethyl acetate, and decomposed by passing dry gaseous NH₃ through its chilled solution in chloroform. After removal of the precipitate the chloroform solution was concentrated in vacuo and the lecithin was precipitated with acetone. It formed a slightly yellow plastic mass. Analysis, found, P 3.9, N 1.9, P:N = 1:1.1.

Sphingomyelin—In a few experiments pure sphingomyelin with a P:N ratio of 1:2 was used, which had been isolated from the spleen of a case of Niemann-Pick’s disease.

Salmine Sulfate—The preparation used was placed at our disposal by E. R. Squibb and Sons, New Brunswick, New Jersey. After purification by precipitation with alcohol from an aqueous solution it formed a white powder. Analysis, found, C 38.1, H 6.5, N 17.9, amino N 10.1, S 6.5.

Chargaff, E., unpublished data.
The aqueous emulsions of the phosphatides used were obtained by treating the material in a mortar with a small amount of water, until a homogeneous emulsion was obtained, followed by gradual dilution with more water and filtration through a thick layer of cotton. In this manner very stable emulsions could be obtained.

To an emulsion of 3.07 gm. of cephalin, Fraction C-1, in 125 cc. of water a solution of 1.0 gm. of salmine sulfate in 50 cc. of water was added. A voluminous white precipitate separated immediately, which was filtered off and repeatedly washed with water and acetone. The product, which weighed 2.86 gm., formed an almost white powder which was soluble in moist ether, chloroform, and warm ethyl acetate and glacial acetic acid. On contact with water, in which it is insoluble, it formed a rubber-like elastic mass. This substance, like all the other protamine-cephalin complexes here described, had no true melting point. When slowly heated in a capillary tube, it softened at about 140° and gradually decomposed to form a dark brown, viscous oil. None of the compounds described contained sulfur. Analysis, found, P 2.9, N 4.7, P:N = 1:3.5. The cephalin content of this preparation may be computed to be 84 per cent on the basis of the phosphorus value and 85.2 per cent on the basis of the nitrogen value.

A solution of 1.0 gm. of this substance in ether was repeatedly shaken with dilute sulfuric acid. The ethereal solution was washed with water, dried over Na&OJ, and concentrated to a small volume. After precipitation with acetone 0.72 gm. of a white powder was obtained which had the same properties as the original product. Analysis, found, P 3.1, N 5.1, P:N = 1:3.6.

From a filtered solution of 500 mg. of the original product in 20 cc. of warm chloroform 440 mg. of a practically unchanged substance were obtained by precipitation with acetone. Analysis, found, P 3.1, N 5.0, P:N = 1:3.5.

The cephalin-salmine complex can also be recovered unchanged from hot ethyl acetate. A filtered solution of 300 mg. of the substance in 15 cc. of hot ethyl acetate on cooling yielded 240 mg. of a slightly yellow amorphous powder. Analysis, found, P 3.0, N 4.6, P:N = 1:3.4. This complex contained 85 per cent of cephalin according to both the nitrogen and phosphorus values.
In order to ascertain whether the relative proportion of cephalin and salmine materially affected the composition of the final product, in another experiment the complex was prepared with an emulsion of 1.0 gm. of cephalin, Fraction C-1, and 1.0 gm. of salmine in a total volume of 100 cc. of water. The product obtained weighed 0.95 gm. Analysis, found, P 2.8, N 5.9, P:N = 1:4.7. This complex contained 80.5 per cent of cephalin on the basis of its phosphorus value and 79.1 per cent according to the nitrogen value.

From another cephalin preparation, Fraction C-3, a similar substance was obtained. From 125 cc. of a 1.3 per cent cephalin emulsion and 25 cc. of a 2.2 per cent salmine solution, 1.61 gm. of the complex were prepared. Analysis, found, P 2.9, N 6.5, P:N = 1:5. The composition of this compound was likewise not changed by treatment with acids or recrystallization.

The experiments here described tend to demonstrate the remarkable stability of the complexes between cephalin and salmine.

*Lecithin or Sphingomyelin and Salmine*—A number of experiments with lecithin and salmine in varying proportions, as described above for cephalin, did not lead to any definite compounds. Several hours after the addition of the protamine solution to the lecithin emulsion a slight flocculation had taken place. The fine floccules were centrifuged off. After repeated washing with water, in which they could be easily emulsified, and treatment with acetone, unchanged lecithin was recovered.

Because of the limited amount of pure sphingomyelin at our disposal, only a few orienting experiments were carried out. These showed, however, that sphingomyelin was not precipitated by the addition of salmine.

*Influence of pH on Precipitation of Phosphatide-Salmine Complexes*—In order to study the formation of the compounds between phosphatides and salmine at different hydrogen ion concentrations, a series of experiments in buffer solutions was carried out. Between pH 1.92 and 5.96 citrate buffers were used; between pH 6.97 and 9.18 phosphate buffers; for pH 10.1 and 11 ammonia-ammonium chloride buffers were employed. The results are reproduced in Table I. As control, the behavior of lecithin emulsions at different pH levels without the addition of salmine was examined. The results, reproduced in Table II, are
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**Table I**

*Reaction between Phosphatides and Salmine at Various pH Levels*

Each tube contained 10 mg. of the phosphatide emulsified in 0.5 cc. of water, 0.5 cc. of the buffer, and 5 mg. of salmine dissolved in 0.5 cc. of water.

<table>
<thead>
<tr>
<th>pH</th>
<th>Cephalin, immediately after addition of salmine</th>
<th>Lecithin</th>
<th>80 min. after addition of salmine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immediately after addition of salmine</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
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<td>3.0</td>
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<td>3.9</td>
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<td>8.0</td>
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<tr>
<td>9.2</td>
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<td>10.1</td>
<td>+</td>
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<tr>
<td>11.0</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+ = heavy precipitate which could not be reemulsified. × = fine flocculation, easily reemulsified. - = no flocculation.

**Table II**

*Stability of Lecithin Emulsions at Various pH Levels*

Each tube contained 10 mg. of lecithin emulsified in 1 cc. of water and 0.5 cc. of the buffer.

<table>
<thead>
<tr>
<th>pH</th>
<th>Lecithin</th>
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<tbody>
<tr>
<td></td>
<td>After 40 min.</td>
</tr>
<tr>
<td>1.9</td>
<td>-</td>
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<tr>
<td>3.0</td>
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<tr>
<td>3.9</td>
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<td>6.0</td>
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<td>10.1</td>
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<tr>
<td>11.0</td>
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</tbody>
</table>

× = fine flocculation, easily reemulsified. - = no flocculation.
in general agreement with the findings of Remesow (22). In the experiments recorded in Tables I and II no additional changes were observed in repeated readings during a period of 18 hours. Emulsions of cephalin alone were stable at all pH levels studied.

These experiments showed clearly that cephalin reacted with salmine within the entire range between pH 2 and 11, lecithin only at pH 10 and 11. At lower pH levels, the addition of salmine to an emulsion of lecithin seems merely to decrease the

**TABLE III**

*Reaction between Phosphatides and Egg Albumin at Various pH Levels*

Each tube contained 10 mg. of the phosphatide emulsified in 0.5 cc. of water, 0.5 cc. of the buffer, and 5.3 mg. of egg albumin dissolved in 0.5 cc. of water.

<table>
<thead>
<tr>
<th>pH</th>
<th>Cephalin, immediately after addition of egg albumin</th>
<th>Lecithin</th>
<th>80 min. after addition of egg albumin</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Immediately after addition of egg albumin</td>
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<tr>
<td>1.9</td>
<td>+</td>
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<td>3.0</td>
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<td>X</td>
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</table>

+ = heavy precipitate which could not be reemulsified. X = fine flocculation, easily reemulsified. - = no flocculation.

stability of the emulsion. It should perhaps be mentioned that at the concentrations studied salmine itself did not show any spontaneous precipitation.

Precipitation of Phosphatide-Egg Albumin Complexes—In these experiments highly purified egg albumin was used. This material which had been twelve times recrystallized was kindly placed at our disposal by Dr. M. Heidelberger. The experiments carried out were similar to those with salmine described in the preceding paragraphs. From the results summarized in Table III it will be seen that cephalin immediately formed characteristic
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precipitates with egg albumin at pH 2, 3, and 4, whereas with lecithin the same type of slow flocculation of the emulsions occurred as in the control experiments reproduced in Table II.

The author is indebted to E. R. Squibb and Sons, New Brunswick, New Jersey, for the sample of salmine sulfate. He wishes to thank Mr. W. Saschek for numerous microanalyses, and Mr. Bernard Kress for general assistance.

SUMMARY

The preparation of compounds between cephalin and salmine and the properties of these substances are described. The significance of the findings with regard to the problem of lipoproteins is discussed.

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