COMPLETE FRACTIONATION OF BRAIN CEPHALIN: ISOLATION FROM IT OF PHOSPHATIDYL SERINE, PHOSPHATIDYL ETHANOLAMINE, AND DIPHOSPHOINOSITIDE

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Brain cephalin (1) has been the name given to a fraction of brain phosphatides characterized by its solubility in ether and insolubility in ethyl alcohol. For many years it was assumed to be a single compound with the chemical structure of diacylglycerylphosphoryl ethanolamine. Work from this laboratory has shown brain cephalin to be a mixture (2). From it were separated two pure phosphatides; namely, phosphatidyl ethanolamine and phosphatidyl serine. The former phosphatide has the chemical structure classically attributed to brain cephalin and the latter has been found to be oleylstearylglycerylphosphoryl serine (3). Besides these two phosphatides, a material was obtained which appeared to be a mixture and to which the name of inositol phosphatide was given for descriptive purposes, since it had inositol as a constituent. This paper contains detailed proof that inositol phosphatide is a mixture and describes a method for the separation from it of phosphatidyl ethanolamine, phosphatidyl serine, and a new inositol-containing phosphatide to which the name of diphosphoinositide has been given. Results establishing in part the chemical structure of diphosphoinositide have already been reported in a preliminary note (4) and are given in detail in the accompanying paper (5).

The evidence here reported establishes that brain cephalin is a mixture of the three phosphatides mentioned above. It contains only small amounts of other substances which can be considered as contaminants. These contaminants are either lipides of the carbohydrate-containing type or non-lipide water-soluble substances among which phosphates appear to be predominant. The water-soluble contaminants can be removed by dialysis.

Brain cephalin, as defined by its solubilities, does not account for all of the lipide NH₂-N¹ present in brain. Not an inconsiderable amount of

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¹ The term NH₂-N is used to indicate aliphatic amino nitrogen determinable by the nitrous acid method of Van Slyke (6).
lipide NH$_2$-N can be found in the supernatant solutions obtained in the course of preparation of brain cephalin. This lipide NH$_2$-N appears to be phosphatidyl ethanolamine, which is to be expected since this phosphatide is soluble in alcohol (2). On the other hand, brain cephalin appears to account for most of the carboxyl N present in brain. Only small amounts of lipide carboxyl N are found in brain lipide fractions other than brain cephalin.

The method of fractionation of the material previously called inositol phosphatide into its different components is the result of many unfruitful attempts. In the earlier part of the work we were handicapped by our lack of knowledge of the chemical nature of the components of the mixture. This prevented the setting up of reliable criteria for the value of the different methods of fractionation that were attempted. Inositol content of the fractions, which was the only way of estimating progress towards isolation of the pure inositol-containing phosphatide, can only be determined by bioassay. This bioassay could only show fairly large differences in inositol content of different fractions obtained. As will become apparent to the reader, the method of fractionation finally developed yields fractions of different inositol content, but the differences at each step are so small that inositol determinations by bioassay would fail to show it. A more reliable criterion was finally provided by the isolation of inositol metaphosphate from inositol phosphatide (described in the accompanying paper (5)), since this finding allowed the assumption that the pure inositol-containing phosphatide would show a phosphorus content larger than that found in known phosphatides. With this idea in mind, fractions obtained from inositol phosphatide by the different methods of fractionation tried were analyzed for phosphorus. The use of the phosphorus content as a criterion led to the fractionation method described below.

This method is based on the fact that diphosphoinositide is less soluble in methyl alcohol than either phosphatidyl serine or phosphatidyl ethanolamine. This difference in solubility is less evident when mixtures are being dealt with. Therefore, it is necessary to repeat the treatment with methyl alcohol a number of times before essentially pure diphosphoinositide is obtained. From the filtrates it is easy to separate phosphatidyl serine and phosphatidyl ethanolamine by means of their different solubility in ethyl alcohol (2).

Phosphatidyl serine and phosphatidyl ethanolamine have been identified by isolating among their cleavage products L-serine from the former and ethanolamine from the latter. Glycerophosphoric acid had been isolated

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2 The term COOH-N or carboxyl nitrogen is used to indicate aliphatic amino acid nitrogen determinable by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (7).
previously from inositol phosphatide (2). Therefore, the two compounds appear to be identical with, or closely related to, those isolated from brain cephalin by a former method (3). Phosphatidyl serine isolated from inositol phosphatide is mainly in the form of a sodium salt, in contrast with that obtained earlier (3) which is mainly in the form of a potassium salt.

EXPERIMENTAL

Analytical Methods—The methods used are described elsewhere (2, 3).

Preparation of Inositol Phosphatide—Cephalin is prepared from beef brain by the method described earlier (2). It is then fractionated by the chloroform-alcohol method (2), modified as described below.

1 gm. of cephalin is dissolved in 8 cc. of CHCl₃ and to the clear solution are added 11.8 cc. of ethyl alcohol (1.45 as much alcohol as chloroform, by volume). A turbidity develops, and on standing, or by centrifugation, the mixture resolves itself into a viscous underlayer (inositol phosphatide fraction) and a clear supernatant solution. The supernatant solution (which contains most of the phosphatidyl serine and phosphatidyl ethanolamine present in the original cephalin) is decanted and the viscous underlayer is treated with ethyl alcohol. A solid precipitate that forms is collected on a Büchner funnel and dried.

The yield of inositol phosphatide is about 5 gm. per kilo of initial tissue. The preparation corresponds to the combination of Fractions I and II as obtained by the chloroform-ethanol method first described (2). The composition of these fractions has already been given (Table I, Columns 2 and 3 (2)). The preparation contains all the inositol present in the starting cephalin. It is, however, a complex mixture which on analysis is found to contain C 53.0 per cent, P 5.38 per cent, inorganic P 1.02 per cent, total N 1.36 per cent, NH₃-N 1.33 per cent, COOH-N 0.87 per cent, carbohydrate (as galactose) from 0.7 to 1.3 per cent, Na 2.04 per cent, K 3.60 per cent, Ca 0.15 per cent, Mg 0.45 per cent.

Fractionation of Inositol Phosphatide by Chloroform-Methanol Method

The method to separate from inositol phosphatide its different components is as follows:

The whole procedure is run at about 4°. 1 gm. of inositol phosphatide is dissolved in 12 cc. of chloroform and to the clear solution are added 22 cc. of methanol. A precipitate forms immediately. The mixture is shaken in a shaking machine for 30 minutes, after which the precipitate separates easily, leaving a water-clear supernatant solution. The precipitate is collected on a Büchner funnel or by centrifugation and is transferred back to the container in which the precipitation was carried out. The precipitate is redissolved in 12 cc. of chloroform (part of the chloroform can be
used to return the precipitate to the original container), 22 cc. of methanol are added to the clear solution, and the procedure repeated exactly as before. The whole operation is repeated for as long as the supernatant solution contains material which on analysis is found to contain <4.5 per cent P. The supernatant solution must be clear, as any cloudiness is due to suspended diphosphoinositide. Material recovered from cloudy supernatant solutions is found to contain on analysis from 4.5 to 7 per cent P. Occasionally it happens that the chloroform-methanol-insoluble precipitate does not go completely into solution in chloroform. The chloroform-insoluble material can neither be removed by centrifugation nor by filtration. It can be eliminated by adding 2 volumes of ethyl ether to the turbid chloroform solution and collecting by centrifugation whatever insoluble material there is. In one case, in which it was studied, the insoluble material represented a very small fraction of the total preparation and had the composition of phosphatidyl serine. The residue obtained on evaporation in a vacuum of the clear chloroform-ethyl ether solution was completely soluble in chloroform. The chloroform-methanol treatment was then resumed.

It has been found that the procedure runs more smoothly and requires a smaller number of successive precipitations when the original inositol phosphatide has not been freed of water-soluble impurities by dialysis. With the non-dialyzed inositol phosphatide, twelve successive precipitations usually suffice, whereas, with the inositol phosphatide that has been purified by dialysis, as many as thirty successive precipitations are required.

The different supernatant solutions are labeled IP1, IP2, and so on, in the order in which they are obtained. In all cases the solvents are removed by evaporation in a vacuum. Therefore, from the original inositol phosphatide there are obtained a number of IP fractions and a final precipitate which contains all of the diphosphoinositide grossly contaminated, especially with inorganic phosphates. The yield of impure diphosphoinositide is about 20 per cent of the original inositol phosphatide, or 1 gm. per kilo of starting brain tissue.

Data on yields for different fractions and results of their chemical analyses are given in Table I.

Study of IP Fractions

From data given in Table I it appears that all IP fractions contain about 3.5 per cent P and 1.4 per cent N, the higher values for P shown by fractions IP9 to IP12 being due to contamination by inorganic phosphates. Practically all of the nitrogen is NH4-N. Otherwise, the fractions appear to fall into three groups: (1) fractions IP1 and IP2 that contain only part of
their NH₂-N as carboxyl N; (2) fractions from IP₄ to IP₈ that have all of their NH₂-N as carboxyl N; and (3) fractions from IP₉ on that appear to be the same as fractions IP₃ to IP₆ contaminated with inorganic phosphates and, possibly, diphosphoinositide. The study of these fractions detailed below establishes that fractions IP₁ and IP₂ are mixtures of phosphatidyl serine and phosphatidyl ethanolamine, fractions IP₃ to IP₈ are phosphatidyl serine, and fractions from IP₉ on are also phosphatidyl serine.

### Table I

Fractionation of Inositol Phosphatide by Chloroform-Methanol Method. Composition of Starting Inositol Phosphatide, of Successive Chloroform-Methanol-Soluble Fractions (Supernatants IP₂), and of Diphosphoinositide Finally Obtained Therefrom

<table>
<thead>
<tr>
<th>Material*</th>
<th>Yield, per cent of starting material of inositol phosphatide</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Inositol phosphatide</td>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td>IP₁</td>
<td>28.4</td>
<td>5.38</td>
</tr>
<tr>
<td>IP₂</td>
<td>17.6</td>
<td>3.60</td>
</tr>
<tr>
<td>IP₃</td>
<td>7.9</td>
<td>3.64</td>
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<td>IP₄</td>
<td>2.00</td>
<td>4.62</td>
</tr>
<tr>
<td>IP₅</td>
<td>0.70</td>
<td>5.56</td>
</tr>
<tr>
<td>Diphosphoinositide</td>
<td>19.0</td>
<td>11.06</td>
</tr>
</tbody>
</table>

* Fractions IP₄ to IP₈ had the same composition as IP₂. The yields were, respectively, 6.1, 4.8, 3.6, 3.9, and 2.7 per cent of starting inositol phosphatide. Fractions IP₁₀ and IP₁₁ had compositions ranging between those of IP₇ and IP₁₂. The yields were, respectively, 1.1 and 0.8 per cent of starting inositol phosphatide.

† All fractions from IP₁ to IP₁₂ had all of their N as NH₂-N.

**Fractionation of IP₁ and IP₂ Fractions and Identification of Phosphatides Separated Therefrom**—From these fractions, either singly or combined, two fairly pure phosphatides can be obtained, one which contains all of its N as NH₂-N in non-amino acid form (lowcarboxyl N phosphatide), and a second which contains all of its N as carboxyl N (high carboxyl N phosphatide).

The method of separation is based on the fact that the former phosphatide is much more soluble in ethyl alcohol than the latter (2).

17.6 gm. of combined IP₁ and IP₂ were dissolved in 160 cc. of chloroform and 600 cc. of ethyl alcohol were added to the clear solution. On standing overnight in the ice box, a precipitate separated which was collected and dried. It weighed 12.6 gm. (71.6 per cent of starting material) and on analysis was found to contain P 3.5 per cent, N 1.35 per cent, carboxyl N 1.32 per cent (high carboxyl N phosphatide).
Material left in the supernatant solution was recovered by removal of the solvent by vacuum distillation. After drying to constant weight, the material obtained weighed 5 gm. (28.4 per cent of starting material) and on analysis was found to contain P 3.5 per cent, N 1.4 per cent, NH₃-N 1.4 per cent, carboxyl N 0.15 per cent (low carboxyl N phosphatide).

Isolation of Ethanolamine from Low Carboxyl N Phosphatide—2.0 gm. of low carboxyl N material obtained as described above were emulsified in 150 cc. of H₂O. Concentrated HCl was added to the chilled emulsion until the precipitation of the phosphatide appeared to be complete. 70 cc. of concentrated HCl were required. This is in sharp contrast to the behavior of the high carboxyl N phosphatide which precipitates quantitatively out of emulsion with a concentration of HCl below 0.1 N. The precipitate was removed by centrifugation, washed once with 220 cc. of 4 N HCl, and next hydrolyzed by boiling 4 N HCl under a reflux for 2 hours. After cooling, the hydrolysate was filtered and the filtrate was evaporated to dryness in a vacuum. The dry residue was taken up in 35 cc. of water, and the solution was treated with an excess of 25 per cent aqueous solution of neutral lead acetate. The insoluble lead salts were removed by filtration and the filtrate treated with H₂S. The lead sulfide that formed was removed by filtration and the filtrate was evaporated to dryness in a vacuum. The residue weighed 250 mg. and contained 18.6 mg. of NH₃-N.

The picrolonate was prepared by dissolving the theoretical amount of picrolonic acid required (350 mg.) and the residue in 30 cc. of boiling water (8). About one-tenth of the picrolonate formed proved insoluble, despite prolonged stirring. The insoluble material was allowed to settle and the clear supernatant was decanted into another tube and let cool slowly. After standing overnight in the ice box, the crystals that had formed were collected. After drying, the crystals weighed 350 mg. and on analysis were found to contain 3.27 per cent NH₃-N and 0.06 per cent carboxyl N. The crystals were recrystallized twice from boiling water without significant change in the NH₃-N content. They were then washed with ether. After drying, they were found to contain 3.95 per cent NH₃-N; theory for ethanolamine picrolonate, 4.31 per cent.

This material was combined with that recovered from the supernatant solutions of the two recrystallizations. The combined material was treated with hot dilute HCl, which resulted in precipitation of most of the picrolonic acid. The filtrate from the picrolonic acid precipitate was freed of residual picrolonic acid by shaking with ether. The water-clear dilute hydrochloric acid solution was evaporated to dryness in a vacuum and a crystalline residue was obtained which weighed 70 mg. On analysis it was found to contain NH₃-N 14.25 per cent, carboxyl N 0.00 per cent, and C 24.6 per cent. Theory for ethanolamine hydrochloride is NH₃-N 14.36
per cent and C 24.6 per cent. Allowing for the aliquots used for analysis, the amount of ethanolamine hydrochloride isolated accounted for over 50 per cent of non-amino acid NH\textsubscript{2}-N in the starting phosphatide.

Isolation of L-Serine from High COOH-N Phosphatide—3.7 gm. of this phosphatide were freed of bases by emulsification in 200 cc. of water and precipitation of the phosphatide by addition of HCl up to 0.1 N concentration. The precipitate was washed once with 0.1 N HCl and next hydrolyzed by boiling 6 N HCl under a reflux for 3 hours. After cooling, the hydrolysate was filtered and the filtrate dried by evaporation in a vacuum. The residue was dissolved and the solution was decolorized with charcoal. The filtrate from the charcoal was found to contain 35.4 mg. of carboxyl N. The solution was concentrated to a volume of 11 cc. and to it were added 660 mg. of p-hydroxyazobenzene-p-sulfonic acid (9), which was dissolved by heating in the boiling water bath. Crystals that formed on standing overnight in the ice box were collected. After drying, they weighed 700 mg. The crystals were dissolved in water, the p-hydroxyazobenzene-p-sulfonic acid was precipitated by addition of excess neutral lead acetate, and the insoluble lead salt removed by filtration. The filtrate was treated with charcoal to remove the last traces of p-hydroxyazobenzene-p-sulfonic acid and the filtrate from the charcoal was treated with H\textsubscript{2}S. The filtrate from the lead sulfide was concentrated to a volume of 3 cc. and treated with 40 cc. of ethyl alcohol. On standing overnight, sharp needles separated. The crystals were collected and, after drying at 100°, weighed 140 mg. On analysis they proved to be L-serine. They accounted for over 60 per cent of the carboxyl N in the starting phosphatide. Results of analyses were as follows:

\[
\begin{array}{l}
C_3H_7O_2N. \text{ Calculated.} \\
\quad \text{C } 34.2, \text{ H } 6.67, \text{ N } 13.32, \text{ carboxyl N } 13.32 \\
\quad \text{Found (corrected for } 0.4\% \text{ ash).} \\
\quad \text{C } 34.2, \text{ H } 6.62, \text{ N } 13.27, \text{ carboxyl N } 13.21
\end{array}
\]

Rotation—A solution in 1 N HCl containing 50 mg. of crystals per cc. showed in a 1 dm. tube a rotation of +0.70° with sodium light; \([\alpha]_D^{20} = +14.2°\). Fischer and Jacobs give +14.5° (10).

Isolation of L-Serine from Combined IP\textsubscript{3} to IP\textsubscript{5} Fractions—By the method described for the high carboxyl N phosphatide, 124 mg. of analytically pure L-serine were obtained from 3 gm. of combined IP\textsubscript{3} to IP\textsubscript{5} fractions. This amount accounted for 43 per cent of carboxyl N present in the original material.

Isolation of L-Serine from Combined IP\textsubscript{10} to IP\textsubscript{12} Fractions—From 2.5 gm. of this material, 90 mg. of analytically pure L-serine were obtained by the method described above. This amount of pure L-serine accounted for 48 per cent of carboxyl N present in the original material.
Fractionation of Brain Cephalin

Bases Combined in Phosphatidyl Serine Separated from Inositol Phosphatide

Fractions IP₂, IP₅, IP₆, and IP₈ were analyzed for Na, K, Ca, and Mg. They were found to contain an average of 1.7 per cent Na and 1.6 per cent K and to be free of Ca or Mg. The ratio, equivalents of total base to atoms of P, was in all cases about 1.00. In comparison, phosphatidyl serine separated from the original cephalin (3) contains about 3 per cent K and between 0.5 and 1.00 per cent Na.

SUMMARY

Brain cephalin has been found to be a mixture of phosphatidyl serine, phosphatidyl ethanolamine, and a new inositol containing phosphatide which is called diphosphoinositol.

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