Preparation of Hydrophilic Colloids of Brain Kephalsins

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Phospholipids can be extracted from certain plant materials as a completely clear aqueous solution (Thierfelder & Klenk, 1930). However, phospholipids usually give a stable translucent emulsion with water. We have found that translucent, water emulsions of inositol phosphatide fraction (Folch's fraction I) or phosphatidylserine (Folch's fraction III) from brain tissue (Folch, 1942) quickly become transparent if ethylenediaminetetra-acetate is added at pH 10. The clear solution is stable even after removal of the reagents by dialysis.

MATERIALS AND METHODS

Brain-kephalin fractions. Phosphoinositide fraction (fraction I), phosphatidylserine (fraction III) and phosphatidylethanolamine (fraction V) were prepared from ox brains by the method of Folch (1942). Fraction I, before dialysis, contained 5.36% of P, 1.18% of N (P/N, 2.05) and 21.4% of ash. Fraction III, before dialysis, contained 3.54% of P, 1.55% of N (P/N, 1.03) and 14.3% of ash. After dialysis for 9 days it contained 3.55% of P, 1.50% of N (P/N, 1.07) and 1.44% of carboxyl N (carboxyl N/N, 0.96). Fraction V, before dialysis, contained 3.42% of P, 2.01% of N (P/N, 0.78) and 5.2% of ash. Each lipid sample was examined by paper chromatography in phenol-water (85:15, v/v) by which phosphatidylserine and phosphatidylethanolamine could be separated (Amelung & Böhm, 1954). Non-dialysed samples of fraction I gave a single elongated spot of lipid accompanied by several amino acid spots which were absent from the dialysed sample. Fractions III and V gave only single lipid spots with R_F values of 0.62 and 0.82 respectively.

The lipids were hydrolysed by heating 10–20 mg. with 5 ml. of 6N-HCl in a sealed tube for 20–40 hr. at 105°. The hydrolysate, after removal of excess of HCl, was submitted to one- and two-dimensional chromatography with phenol-water (80:20, v/v), or butanol-acetic acid-water (40:10:10, by vol.) or both of these solvents. In the hydrolysate of the dialysed sample of fraction I, serine and ethanolamine were identified as well as three ninhydrin-reacting spots with the R_F values of aspartic acid, glutamic acid and alanine. Fractions III and V each gave single ninhydrin-positive spots which were identified as serine and ethanolamine respectively. Inositol (Nagai & Kimura, 1958) was detected in fraction I but not in fractions III and V. Choline (Hawke & Lea, 1953) was absent from all three fractions.

Brain-'lecithin' fraction. An ethanol-soluble, acetone-insoluble lipid preparation from ox brain was used as a lecithin-rich fraction.

Reagents for clearing lipid emulsions. Aq. NH_3 soln.- NH_4Cl stock buffer solution (reagent A) was 70 g. of NH_4Cl and 570 ml. of conc. aqueous NH_3 (sp.gr. 0.90) diluted with water to 1 l. Clearing agent at pH 10 (reagent B) was 5 ml. of reagent A added to 50 ml. of aqueous 5% (w/v) solution of ethylenediaminetetra-acetate (EDTA; A.R. disodium dihydrate salt). EDTA solution at pH 10 for dialysis (reagent C) was 1 ml. of reagent A added to 50 ml. of aqueous 0.5% solution of EDTA.

RESULTS

Preparation of a transparent aqueous solution of kephalins. Reagent B was added in portions, with stirring, to a fresh, non-dialysed preparation of fraction I or III or to the conc. aqueous suspensions until the final concentration of lipid was about 3% (w/v). The mixtures soon became clear, as shown in Fig. 1. EDTA and other diffusible substances could be removed from the clear solution by dialysis through cellophan at 4° with constant stirring against 100 vol. of reagent C for 24 hr., then against 100 vol. of diluted reagent A (1:50, v/v; pH 10) for 24 hr. and finally for a total of 5 days against nine changes of 100 vol. of distilled water. A stable clear solution could also be obtained from fraction III simply by dialysing against water immediately after clearing by reagent B.

The pH values of the dialysed transparent solutions were about 3. The pH of the original water-emulsion was about 6.4 in fraction I and 4.6 in fraction III. The dialysed solutions contained much less ash, namely, 1.3% in fraction I and 3.0% in fraction III. The lipid samples which have been recovered by freeze-drying from the dialysed clear solution were submitted to infrared spectral analysis. The characteristic absorptions of EDTA (1630 cm^{-1} , 1400 cm^{-1} , 1367 cm^{-1} , 1317 cm^{-1} , 1290 cm^{-1} and five bands between 960 cm^{-1} and 800 cm^{-1}) were absent. The spectra were closely similar to that of the metal-free kephalins, which are given elsewhere (Kimura & Nagai, 1960).

Stability of the clear solution. The hydrophilic colloids thus obtained were stable under ordinary conditions, but on freezing and thawing the transparent solution from fraction III changed into a gross suspension and that from fraction I into a slightly translucent emulsion. Addition of EDTA

and of ammonia at a suitable pH restored the transparency of the suspension from fraction III, but the emulsion from fraction I remained turbid. Even with fraction III, however, several repetitions of the same treatment, freezing, thawing and clearing, brought about an irreversible change into suspension. This seemed to be reflected in the behaviour of the lipid. Dialysed fraction I gave a clear solution by the EDTA treatment in the same way as the undialysed material, but the resulting solution changed irreversibly into a turbid emulsion if it was dialysed to remove EDTA. This turbidity caused by removal of EDTA was not observed with fraction III.

The clear aqueous lipid solutions remained transparent on the addition of alkali but immediately become turbid on the addition of neutral salts such as sodium, potassium or ammonium chloride. The turbid solution could be cleared again by adding EDTA and alkali. Shaking with chloroform and

ether also caused turbidity. Lipid preparations recovered from the clear aqueous solutions by freeze-drying showed no marked change in solubility behaviour. They were easily soluble in chloroform and formed translucent or opaque emulsions with water.

Clearing action of ammonia. In the absence of EDTA, aqueous ammonia had a clearing action but only when sufficient ammonia was added to the aqueous emulsion of lipids to bring the pH to 10.8. The resulting clear solution was far less stable than that obtained by the use of EDTA. The clear aqueous solution of fraction III remained so after removal of ammonia, but the turbidity caused by adding neutral salts or by freezing and thawing did not disappear permanently on the addition of ammonia, even if transient clarification occurred. When the preparation of lipid had been purified by dialysis against water for 9 days, the clear aqueous solution, obtained by adding ammonia, became

Fig. 1. Clearing effect of EDTA on aqueous emulsions of brain kephalins. 1, Plain water; 2, water emulsion of fraction I (30 mg. of lipid/ml.); 3, Aq. NH_3 soln.- NH_4Cl buffer (pH 10) emulsion of fraction I (30 mg. of lipid/ml.); 4, transparent solution obtained by adding EDTA to the buffered emulsion (3); 5, unchanged transparent solution obtained after dialysis of the solution (4) against water; 6, plain buffer solution of aq. NH_3 soln.- NH_4Cl (pH 10); 7, aq. NH_3 soln.- NH_4Cl buffer (pH 10) emulsion of fraction III (20 mg. of lipid/ml.); 8, transparent solution obtained by adding EDTA to the buffered emulsion (7); 9, unchanged transparent solution obtained after dialysis of the solution (8) against water (8 mg. of lipid/ml.); 10, aq. NH_3 soln.- NH_4Cl buffer (pH 10) emulsion of fraction V (30 mg. of lipid/ml.); 11, buffered emulsion (10) with addition of EDTA showing no clearing. (Test tubes are approx. 5 mm. in diameter.)

turbid again on removing the ammonia by dialysis. A translucent emulsion of fraction I also becomes transparent when ammonia was added, but it soon became very turbid again, and on standing a white precipitate formed with a clear supernatant which became turbid on dialysis. The white precipitate contained inorganic phosphate and lipids.

SUMMARY

1. When a turbid water emulsion of inositol phosphatide fraction (Folch's fraction I) or phosphatidylserine (Folch's fraction III) from brain tissues is added to a solution of ethylenediaminetetra-acetate at pH 10, the solution quickly becomes transparent and remains so even after removal by dialysis of the reagents added. Such a phenomenon is not seen with phosphatidylethanolamine (Folch's fraction V) or with a brain-'lecithin' fraction.

2. The clear solutions become turbid on freezing

and thawing, and on the addition of chloroform, ether or neutral salts.

3. Aqueous ammonia also has a clearing action at higher pH values, but the resulting clear solutions were far less stable than those obtained by the use of ethylenediaminetetra-acetate.

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Infrared Spectra of Brain Phosphatidylserine

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Infrared spectroscopy has been applied to many complex lipids, but the infrared spectrum of phosphatidylserine seems not to have been published. Marinetti, Erbland & Stotz (1958) mention that a sodium salt of brain phosphatidylserine has more absorption at $6.1\ \mu$ and less at $2.9\ \mu$ than has the free acid form.

MATERIALS AND METHODS

Phosphatidylserine. This was prepared as described by Kimura & Nagai (1960).

Metal-free phosphatidylserine. This was prepared according to the procedure of Folch (1948).

Monosodium phosphatidylserine. In this the atomic ratio Na:P was 1:1; it was prepared by the addition, with cooling and stirring, of the calculated amount of CO_2 -free NaOH (0.01 N) to a suspension (2%, w/v) of the metal-free phosphatidylserine in CO_2 -free water. The solution was then freeze-dried.

Disodium phosphatidylserine. In this the atomic ratio Na:P was 2:1 and it was prepared similarly.

Infrared spectroscopy. All infrared spectra were obtained by the KBr-disk technique. A Hitachi Model EPI-2 double-beam automatic-recording spectrophotometer (Hitachi Ltd., Tokyo, Japan) with an NaCl prism was used. The

same infrared spectral patterns were obtained in a KBr disk, in chloroform solution and in Nujol film. When phosphatidylserine was dialysed against water for 9 days, its ash content was decreased and, in parallel with this, a similar spectral pattern to that of the metal-free form was obtained.

RESULTS AND DISCUSSION

The infrared spectra of the four forms of phosphatidylserine are shown in Fig. 1. All forms possess the following bands which can be correlated with specific molecular groups: $1750\text{--}1740\ \text{cm}^{-1}$, C=O ester, stretching; $1475\ \text{cm}^{-1}$ and $1375\ \text{cm}^{-1}$, CH deformation; $1235\ \text{cm}^{-1}$, P=O free, stretching; and $1050\ \text{cm}^{-1}$, P—O—C stretching. Four absorption bands ($1655\ \text{cm}^{-1}$, $1525\ \text{cm}^{-1}$, $1420\ \text{cm}^{-1}$ and $1175\ \text{cm}^{-1}$) are affected by the form of the material and thus must be attributed to the acidic ($-\text{CO}_2\text{H}$, $\text{—}\overset{\text{||}}{\text{P}}\text{—OH}$) and basic ($-\text{NH}_2$) groups of phosphatidylserine.

The $1655\ \text{cm}^{-1}$ absorption, a characteristic of the original phosphatidylserine, vanishes in the metal-free form but reappears in the mono- and disodium forms, although the bands become broader