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Studies on Phospholipids

6. A COMPLEX PHOSPHOLIPID FROM SHEEP BRAIN*

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The occurrence of complex phospholipids in rat liver and sheep brain has been described (Collins, 1959) and it was shown that, after *N*-2:4-dinitrophenylation and methylation, three forms could be distinguished with partition coefficients of 1.0, 2.5 and 8 in carbon tetrachloride-methanol-water. The material with a partition coefficient of 1.0 predominated in sheep brain but was present in small amounts only in other tissues (Collins, 1959). The present paper describes the isolation and purification of this complex phospholipid from sheep brain. A preliminary account has already been published (Collins, 1958).

METHODS

The analytical methods used were those described by Wheeldon & Collins (1957) for amino N, Wheeldon &

Collins (1958) for choline, Collins & Wheeldon (1958) for ethanolamine and serine, Stern & Shapiro (1953) as adapted by Collins (1959) for fatty acids, Beadle (1944) for inositol, Collins (1959) for complex phospholipids, Park & Johnson (1949) for reducing sugars and Feulgen & Grünberg (1939) for fatty aldehydes.

Phosphorus. A sample of lipid containing between 3 and 25 μ g. of P was added to a micro-Kjeldahl digestion flask and 0.05 ml. of 72% (w/v) perchloric acid added. The mixture was gently refluxed for 20 min., allowed to cool and 5 ml. of water added, followed by 5 ml. of the diluted mixed metavanadate-molybdate reagent described by Michelsen (1957). After 5 min. the extinction at 315 m μ was measured and simultaneous measurements were made with control solutions containing acid and reagent, two without P and two containing a known amount of P.

Hydroxyl groups. A sample containing between 0.5 and 8.0 μ moles of a non-volatile alcohol was treated with 0.25 ml. of acetyl chloride for 10 min. at 30°. The excess of acetyl chloride and the hydrogen chloride liberated during the reaction were removed *in vacuo* and the procedure described by Stern & Shapiro (1953) for ester groups was applied, octadecanol and cholesterol being used as reference substances.

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Nitrogen. The micro-Kjeldahl procedure used by Wheeldon & Collins (1957) was employed, except when nitro groups were present; in these cases the author is indebted to Dr J. Fildes, who estimated them by the reduction procedure described by Friedrich (1933).

Countercurrent distribution. The solvent systems used were aqueous 85% (v/v) ethanol-light petroleum (b.p. 40–60°; Lovern, 1952), light petroleum (b.p. 40–60°)–acetone–propan-2-ol–water (40:10:40:10, by vol.) and carbon tetrachloride–methanol–water (62:35:3:15, by vol.; Cole, Lathe & Ruthven, 1953). The other details were as described by Collins (1959).

Paper chromatography. The following solvent systems were used for the identification of glycerol 1-phosphate: phenol saturated with water, propan-2-ol–aqueous 1% ammonium sulphate solution (2:1, by vol.; Anand, Clark, Hall & Todd, 1952), methanol–aqueous 2N-ammonia solution (70:30, v/v; Burrows, Grylls & Harrison, 1952), acetone–aqueous 35% (v/v) formic acid (60:40, v/v; Burrows *et al.* 1952), butanol–acetic acid–water (4:1:5, by vol.; Partridge, 1948) and propanol–water (4:1, v/v; Olley, 1956). Phosphate esters were detected by the method of Hanes & Isherwood (1949) as modified by Bandurski & Axelrod (1951), and *cis*-glycols by the method of Baddiley, Buchanan, Hondschumacher & Prescott (1956).

The solvent systems described by Partridge (1948) and Olley (1956) were used for the paper chromatography of choline, detected by the method of Levine & Chargaff (1951), and of sugars, detected by the aniline hydrogen phthalate reagent described by Partridge (1949).

For dinitrophenyl (DNP)-ethanolamine and DNP-serine the solvent systems described by Collins & Wheeldon (1958) were used.

Paper electrophoresis. This was carried out in 0.02M-sodium citrate buffer (pH 3.5), at 2 ma, 200 v for 4 hr. and on no. 1 Whatman paper.

Preparation of the lipids. Sheep brain (106 g.) which had been transported in ice from the abattoir was extracted and the lipids (6.8 g.) were washed with acid, treated with 1-fluoro-2,4-dinitrobenzene as described by Wheeldon & Collins (1957) and then with diazomethane (Collins & Wheeldon, 1957). The lipids were divided into a non-polar and a polar fraction by countercurrent distribution in aqueous 85% (v/v) ethanol-light petroleum, with 50 transfers (Collins & Wheeldon, 1957; Collins, 1959).

Hydrolysis. The complex phospholipid (25 or 50 mg.) was refluxed with 5 ml. of anhydrous ethanolic 2N-hydrogen chloride solution for 3 hr., heated *in vacuo* to remove the hydrogen chloride and ethanol and 5 ml. of water added.

When cold the mixture was filtered and the filter washed with water until the washings were colourless. The insoluble material on the filter, and that left in the flask, was dissolved in light petroleum and contained the fatty acids and the fatty alcohols. The aqueous solution was shaken three times with 10 ml. of diethyl ether, which extracted DNP-ethanolamine and DNP-serine. The water-soluble, diethyl ether-insoluble material formed the third fraction.

The light petroleum-soluble portion of the hydrolysate was refluxed with 10 ml. of ethanolic 2N-potassium hydroxide solution for 2 hr. and, after the addition of 10 ml. of water, the solution was extracted three times with 10 ml. of diethyl ether. The ethereal solutions were combined, washed three times with 10 ml. of water and the solvent was removed *in vacuo*. This constituted the unsaponifiable fraction. The combined aqueous solution was acidified (pH 2) and the solution was again extracted three times with 10 ml. of diethyl ether. The combined ethereal extracts were washed three times with 10 ml. of water, the solvent was removed *in vacuo* and the fatty acids were recovered.

Molecular-weight determination. The molecular weight was determined by the isopiestic method described by Signer (1930), with, as reference substance, a sample of peanut oil which contained less than 1% of unsaponifiable material and had a saponification equivalent of 890. In each experiment 70 mg. of the complex phospholipid was dissolved in 1.4–1.8 ml. of diethyl ether and 70 mg. of the peanut oil in 0.5–1.0 ml. of diethyl ether, so that in some experiments the distillation was from the phospholipid solution to the peanut-oil solution and in others the reverse. The mean value for the molecular weight was 2030.

Dr D. D. Perrin measured the depression of the freezing point in cyclohexane; 313 mg./10 ml. gave a depression of 0.36°, equivalent to a molecular weight of 2200.

RESULTS

Isolation and purification of the complex phospholipid derivative

Table 1 shows that the polar fraction of the dinitrophenylated and methylated lipids contained 56% of the amino N. Analysis of this fraction by countercurrent distribution in carbon tetrachloride–methanol–water (Collins, 1959) showed the presence of three components with partition coefficients of 1.0, 2.5 and 8.0 with respectively 0.47, 0.25 and 0.30 m-mole of amino N.

Table 1. *Isolation of a complex phospholipid from sheep brain: analysis of the various fractions*

Fraction	Weight (g.)	P (mg.atoms)	Amino N (mg.atoms)	Amino N/P
(1) Starting material	7.10	3.29	1.87	0.57
Non-polar fraction	4.12	0.78	0.83	1.06
Polar fraction	2.98	2.51	1.04	0.41
(2) After first countercurrent distribution in CCl ₄ –methanol–water	1.17	1.05	0.50	0.48
(3) Final stage, after remethylation and countercurrent distribution in aqueous 85% (v/v) ethanol–light petroleum				
Non-polar fraction	0.34	0.15	0.05	0.3
Polar fraction	0.58	0.57	0.28	0.49

The polar fraction was allowed to stand with 300 ml. of acetone at 0° for 18 hr. (Collins, 1959) and the acetone-insoluble material re-treated with acetone. Three fractions were obtained: acetone-soluble material with $\lambda_{\max}$ at 335 m μ and amino N/P 0.56; material sparingly soluble in acetone with $\lambda_{\max}$ at 343 m μ and amino N/P 0.17; and acetone-insoluble material with $\lambda_{\max}$ at 343 m μ and amino N/P 0.08.

The acetone-soluble material was distributed in carbon tetrachloride-methanol-water with 50 transfers and the contents of tubes 13-39 were combined (see Table 1 for analyses). The countercurrent distribution showed the presence of material with partition coefficient of 3.3, which was not present in the original mixture, and this indicated some decomposition. The material was redistributed in carbon tetrachloride-methanol-water (50 transfers, Fig. 1) and analyses of fractions 13-37 showed that the ratio amino N/P was 0.470 ± 0.008 . These fractions were combined and redistributed in carbon tetrachloride-methanol-water (110 transfers) and the contents of tubes 46-68 were combined. This material, which had amino N/P 0.45, was treated with diazomethane and separated into a non-polar and a polar fraction by distribution in aqueous 85% (v/v) ethanol-light petroleum; the analyses of these two fractions are shown in Table 1, the polar fraction representing the final product.

Fig. 2 shows the countercurrent distribution of the final product in light petroleum-acetone-propan-2-ol-water. Countercurrent distribution of the polar fraction of the dinitrophenylated and

Fig. 1. Countercurrent distribution of 1.17 g. of crude complex phospholipid in CCl_4 -methanol-water; each fraction was diluted to 100 ml. with ethanol and the extinction at 335 m μ measured (—). The theoretical distribution for k 1.0 is shown (---).

methyalted lipids indicated the presence of 0.47 m-mole of the complex phospholipid and, as the final yield was 0.28 m-mole, the overall yield was 59%. In two other experiments the yield was 25%. If it is assumed that the molecular weight of the complex phospholipid before dinitrophenylation is 1884 then the sheep-brain lipids contain about 12% of this material.

Properties of the complex phospholipid derivative

The complex phospholipid was a deep-yellow waxy material very soluble in light petroleum, chloroform, benzene, acetone and ethanol but insoluble in water. The compound was unstable if kept in solution at room temperature and in the light and after 6-7 days gave rise to about 10% of products with partition coefficients in carbon tetrachloride-methanol-water of 1.5 and several with values in excess of 2.0. All preparations were therefore stored as solids at -15° .

The minimum molecular weight from the P content was 1030 and the determination of the molecular weight by two methods gave values of 2030 (isopiestic) and 2200 (freezing point). It was concluded that there were two atoms of P/molecule

Fig. 2. Countercurrent distribution of 7.85 mg. of the complex phospholipid in light petroleum-acetone-propan-2-ol-water. The contents of each tube were dissolved in 10 ml. of light petroleum and the extinction at 335 m μ was measured (●). ○, Expected values for k 0.60.

and elementary analysis corresponded to the empirical formula $C_{105\pm1}H_{183\pm2}O_{29}N_6P_2$ (Found: C, 61.4, 61.5; H, 8.8, 8.9; N, 4.1, 4.0; P, 3.0, 3.0. $C_{106}H_{185}O_{29}N_6P_2$ requires C, 61.5; H, 8.9; N, 4.1; P, 3.0. $C_{105}H_{183}O_{29}N_6P_2$ requires C, 61.4; H, 8.9; N, 4.1; P, 3.0 %).

The absorption maximum was at 333–335 $m\mu$ in light petroleum and the molecular extinction coefficient (17 200) indicated the presence of one *N*-2:4-dinitrophenyl group/mole, and this accounted for three of the six N atoms.

Qualitative tests showed the absence of free amino groups, aldehydogenic groups and S.

Hydrolysis products of the complex phospholipid derivative

The complex phospholipid was hydrolysed and the products were separated into light petroleum, diethyl ether and water-soluble fractions as described in Methods. Table 2 gives the analyses of these fractions.

The water-soluble, diethyl ether-insoluble portion of the hydrolysate contained less than 0.05 atom of inorganic P/mole and on paper chromatography and electrophoresis only one phosphate spot was obtained, which in every case coincided in R_f with glycerol 1-phosphate and gave a positive test for a *cis*-glycol. In propan-2-ol-aqueous ammonium sulphate solution the R_f of glycerol 1-phosphate was 0.40, of glycerol 2-phosphate was 0.27 and the unknown was 0.39. It was therefore concluded that the unknown material was glycerol 1-phosphate and that, because only one phosphate spot was obtained and there were 2 moles of P/mole, there must be 2 moles of glycerol 1-phosphate/mole.

The amount of the unidentified N-containing compound in the water-soluble fraction was obtained from the difference between the total N (1.95 moles/mole) in this fraction and the amount of choline N. There was no reaction with ninhydrin and the only unidentified material located on

paper chromatograms was an ultraviolet-absorbing spot with R_f 0.25 in propanol-water and 0.38 in butanol-acetic acid-water. On paper electrophoresis at pH 3.5 the movement was 0.68 of that of orthophosphate. In 0.1N-hydrochloric acid λ_{max} was at 257 $m\mu$ and $E_{1cm}^{1\%}$ in three experiments was 5.6, 11.9 and 13.2, based on the amount of complex phospholipid. The variable yield may indicate either that the material is unstable or that it is an artifact derived from a precursor during the hydrolysis.

Further hydrolysis by heating the water-soluble fraction with 6N-hydrochloric acid for 24 hr. at 110° in a sealed tube did not yield any additional choline and a test for inositol was negative.

The portion of the original 3 hr. hydrolysate that was soluble in diethyl ether or light petroleum contained 1.09 moles/mole of unidentified N-containing compound. There was no reaction with ninhydrin or fluorodinitrobenzene and it was concluded that sphingosine was absent.

From 100 mg. of complex phospholipid 22 mg. of unsaponifiable material was obtained with 1.04 hydroxy groups/mole of phospholipid. Chromatography on alumina yielded a fraction, eluted by chloroform, containing all the hydroxy groups and on which the Liebermann-Burchard test for sterols was negative.

Examination of lipids not treated with 1-fluoro-2:4-dinitrobenzene and diazomethane

Hydrolysis of the complex phospholipid had shown the presence in the hydrolysate of an unidentified water-soluble N-containing compound, an ultraviolet-absorbing material which may or may not be identical with the first material and two compounds soluble in organic solvents, a fatty alcohol and another unidentified N-containing compound. The ultraviolet-absorbing material and the fatty alcohol were sufficiently well characterized to warrant a search in lipids not treated with fluorodinitrobenzene and diazomethane to eliminate the possibility that they were artifacts due to the chemical manipulation of the lipids.

With the extraction procedure described by Wheeldon & Collins (1957), 12.03 g. of lipids was obtained from a sheep brain (219 g.) and the acetone-insoluble lipids (9.21 g.) were hydrolysed by heating under reflux with 200 ml. of anhydrous ethanolic 2N-hydrogen chloride solution for 6 hr. The ethanol and hydrogen chloride were removed *in vacuo*, 1 l. of light petroleum was added and the mixture allowed to stand for 18 hr., after which the light petroleum solution was washed three times with 50 ml. of water and the washings were added to the light petroleum-insoluble material. The light petroleum-soluble material (8.04 g.) was washed with N-hydrochloric acid, with the addition of 10 %

Table 2. *Analysis of the hydrolysate of the sheep-brain complex phospholipid*

Water-soluble materials	Moles/mole
Glycerol 1-phosphate	2.0
Choline	0.96
Sugar (as glucose)	0.96
Unidentified N compound (by difference)	0.99
Diethyl ether-soluble materials	
DNP-ethanolamine	0.95
DNP-serine	0.05
Light petroleum-soluble materials	
Fatty acids	1.92
Fatty alcohol	1.04
Unidentified N compound (by difference)	1.09

(v/v) of ethanol to reduce emulsification, and washed with water to remove excess of hydrochloric acid, yielding 6.44 g. This material was hydrolysed by heating on a boiling-water bath with 20 ml. of ethanol and 5 ml. of 60% (w/v) potassium hydroxide solution for 1 hr. The mixture was cooled, an equal volume of water added and extracted three times with 100 ml. of diethyl ether. The combined ethereal solution was washed three times with 10 ml. of water and the solvent removed by heating *in vacuo*, the residue weighing 1.31 g.

This material was chromatographed on alumina (20 g.) and four fractions were collected: (1) eluted by light petroleum, 0.208 g., equivalent weight for one hydroxy group, 920; (2) eluted by chloroform, 0.454 g., equivalent weight for one hydroxy group, 460; (3) eluted by chloroform-ethanol (1:1, v/v), 0.175 g., equivalent weight, 350; (4) eluted by chloroform-ethanol (1:2, v/v), 0.030 g., equivalent weight, 420. Fractions 2, 3 and 4 were combined and the chromatographic separation on alumina (20 g.) was repeated. The material eluted by chloroform weighed 0.445 g. and was a white wax, freely soluble in light petroleum, benzene, chloroform and sparingly soluble in methanol and ethanol. (Found: C, 75.8; H, 11.7. $C_{30}H_{56}O_4$ requires C, 75.0; H, 11.7. $C_{31}H_{56}O_4$ requires C, 75.7; H, 11.4%). The equivalent weights calculated from the determination of hydroxy groups with octadecanol and cholesterol as reference substances were 470 and 477 respectively.

The infrared spectrum measured by Dr E. Spinner showed the absence of any band due to carbonyl groups but did contain bands which were consistent with hydroxy and ether groups.

The OH/P molar ratio of the crude, unsaponifiable material was 0.27 and the final value was 0.14. These figures represent the upper and lower limits to the amount of fatty alcohol in the total phospholipids and, assuming a molecular weight of 475, this corresponds to 4.9-9.2% of the acetone-insoluble lipids.

The light petroleum-insoluble materials obtained after hydrolysis of the sheep-brain lipids not treated with fluorodinitrobenzene and diazomethane were examined by paper chromatography in propanol-water and butanol-acetic acid-water, and an ultraviolet-absorbing spot was present with the same R_f as that obtained with material from the complex phospholipid. The absorption maximum in water was 255 m μ and $E_{1\%}^{1\text{cm}}$ was 0.8 calculated on the total acetone-insoluble lipids.

DISCUSSION

Phosphatidylethanolamine has seldom been isolated in a pure state from animal tissues and then apparently only in low yield. Folch (1942) isolated

phosphatidylethanolamine (amino N/P 0.91; N/P 1.07 and 3.65% of P) from ox brain in a yield of 2.6 μ moles/g. fresh tissue. Chargaff, Ziff & Rittenberg (1942) showed by isotopic dilution that ox brain contained 14 μ moles of ethanolamine-containing lipids/g. of fresh tissue and thus Folch's (1942) yield was about 19% of the total ethanolamine-containing phospholipids. Folch (1942) also isolated from ox brain phosphatidylserine (amino N/P 1.02; N/P 1.00 and 3.58% of P) in a yield of 4.3 μ moles/g. of fresh tissue. Chargaff *et al.* (1942) obtained 6.8 μ moles of serine-containing lipids and thus Folch's yield was 63%. It can be assumed that the complex phospholipid, the isolation of which forms the subject of this paper, represents part of the 81% of the ethanolamine-containing phospholipids which Folch (1942) discarded in his isolation of phosphatidylethanolamine.

The isolation of the complex phospholipid derivative from sheep brain was based on its solubility in cold acetone and on its partition coefficient in carbon tetrachloride-methanol-water (1.0) and, although the distribution curve (see Fig. 1) was broader than that required by theory for a single compound, this is probably due to the fact that a mixture of fatty acids was present. The distribution curve in light petroleum-acetone-propan-2-ol-water shown in Fig. 2 demonstrates that the divergence from theory is not great in this solvent system.

In the complex phospholipid isolated from sheep brain, the molar ratios between the various residues are nearly integral (P, 2; total N, 6.05; amino N, 1.0; choline, 0.96; sugar, as glucose, 0.96; fatty acids, 1.92; fatty alcohol, 1.04), the molecular weight is double that of the known phospholipids and it contains two unidentified N-containing residues, a complex fatty alcohol and a sugar.

The presence of unidentified N-containing compounds in phospholipids has been noted by Hawke (1956), Hanahan, Dittmer & Warashima (1957) and McKibbin (1957), and the present results enable the unidentified N-containing residues to be related to a definite component of the phospholipid mixture, i.e. to a complex phospholipid.

Saito & Akashi (1957) have reported an unidentified base in the hydrolysate of the lipids of *Bacillus alcaligenes faecalis*, which had λ_{max} in 0.1N-hydrochloric acid of 255.5 and 227.5 m μ , R_f 0.30 in butanol-acetic acid-water (4:1:5, by vol.) and on electrophoresis was almost immobile at pH 7.0 and 6.2. Tests with ninhydrin, and for P and sugars, were negative. This material may be identical with the ultraviolet-absorbing substance obtained from the complex phospholipid.

The presence of a fatty alcohol with a molecular weight of 475, one hydroxy group and three ether

linkages is unexpected and provides an explanation of the high solubility in light petroleum of a complex containing so many polar groups. Fatty alcohols are known to occur (Deuel, 1951), such as the triterpenes in wool fat (agosterol $C_{30}H_{48}O$ and lanosterol $C_{30}H_{50}O$), and the glyceryl ethers which occur in many animal tissues (batyl alcohol $C_{21}H_{33}O_3$, chimyl alcohol $C_{19}H_{40}O_3$ and selachyl alcohol $C_{21}H_{42}O_3$), but neither group corresponds with the material isolated from the sheep-brain complex phospholipid. Recently Carter, Smith & Jones (1958) have reported the presence of batyl alcohol in egg phospholipids and thus the occurrence of ethers in phospholipids is probably widespread.

The presence of 2 moles of glycerol 1-phosphate, 1 mole of ethanolamine, 1 mole of choline and 2 moles of fatty acids suggests that lysophosphatidylcholine and lysophosphatidylethanolamine may form the backbone of the molecule. This would be consistent with the predominance of the glycerylphosphoryl-base structure in phospholipids and, because of the instability of the complex phospholipid on chromatography, may explain in part the finding of lysophospholipids in some tissues (Lea, Rhodes & Stoll, 1955). The complex phospholipid may thus consist of a molecule of lysolecithin and a molecule of lysophosphatidylethanolamine joined together by a bridge consisting of the two unidentified N-containing residues, the sugar and the fatty alcohol. The sum of the molecular weights of oleyl-lysolecithin, methyl *N*-2,4-dinitrophenyloleoyl-lysophosphatidylethanolamine, glucose and the fatty alcohol is 1809. The molecular weight of the complex phospholipid is 2030 and, on the assumption that it is formed from the condensation of six residues by the splitting off of five molecules of water, the total weight of the residues will be 2120. The combined molecular weight of the two N-containing compounds will thus be 311 and they will contain six atoms of oxygen.

The precise nature of the complex phospholipid must await further chemical studies but the fact that it is concentrated in brain tissues (Collins, 1959) suggests that it may be a component of myelin.

SUMMARY

1. From sheep brain, a derivative of a complex phospholipid has been isolated with a molecular weight of 2030 and containing the following residues: 2 moles of glycerol 1-phosphate, 1 mole each of dinitrophenylethanolamine, choline, a sugar and a fatty alcohol, 2 moles of fatty acids and two unidentified nitrogen-containing materials.

2. The fatty alcohol had a molecular weight of 475, with one hydroxy group, and constituted 4.9–9.2% of the total phospholipids of sheep brain.

3. One of the unidentified nitrogen-containing residues after hydrolysis was water-soluble and contained neither free amino groups nor quaternary ammonium groups.

4. The complex phospholipid constituted 28% of the total sheep-brain phospholipids.

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