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The large-scale preparation of unsaturated phosphatidyl cholines from egg yolk

The permeability of lipid bilayers has been shown to depend upon the degree of unsaturation of the phosphatidyl cholines used[1]. In order to investigate the permeability properties of bilayers using a system such as the liposome, considerable quantities of phosphatidyl choline were required, with varying degrees of unsaturation. This paper reports how such quantities (0.5–1.0 mmole) of unsaturated phosphatidyl cholines may be prepared.

Two methods, apart from partial synthesis which is only practicable for the less unsaturated phosphatidyl cholines, were considered for the preparation of material particularly rich in $C_{22:6}$ fatty acid chains. The first method involves the separation of phosphatidyl cholines from various sources, and the second method the fractionation of the molecular species obtained from a single source. The second approach was chosen on grounds of reproducibility and experimental convenience.

The preparation of smaller quantities of specific phosphatidyl cholines has been achieved by TINOCO *et al.*[2], and LYMAN *et al.*[3], using a modification of ARVIDSON's[4] procedure. The quantities prepared by this method are insufficient. Use was made of the following two observations: egg yolks can have their unsaturated lipid content increased preferentially by feeding the chickens a diet rich in unsaturated fats[5,6], the process taking about three weeks to reach equilibrium: and when phosphatidyl choline or ethanolamine are subjected to chromatography on silicic acid columns, the peaks obtained are not homogeneous with respect to the fatty acid composition of the individual phospholipid[7]. A method has been developed, making use of these observations, that enables one to prepare phosphatidyl cholines of varying degrees of unsaturation.

Two groups of chickens (Sussex heavy hybrids) were used, so that one group could be used to obtain the more saturated phosphatidyl cholines, and the other group the more unsaturated product. The diet consisted of 90 % (by weight) of a fat free basal diet formulated in keeping with the requirements laid down by the Agricultural Research Council for the feeding of poultry[8] (the help of M. C. Stevenson, Unilever Research, Sharnbrook, Beds. is gratefully acknowledged). The basal diet was supplemented with 10 % (by weight) of either crude tallow (Bird Chemical Works, Duxford, Cambs.) or a mixture of 5 % cod liver oil B.P. and 5 % crude pilchard oil (Van Den Bergh's and Jurgens, Bromborough, Ches.). Butylated hydroxytoluene was added to the fats to prevent oxidation (200 ppm). The fatty acid composition of these two diets, and of the crude egg phosphatidyl cholines prepared one month after starting the experimental diet, are shown in Table I.

Phosphatidyl cholines obtained from the tallow-fed group were used for the preparation of purified phosphatidyl cholines with a $C^{\beta}_{22:6}$ content of 2–10 %, whereas the fish-oil fed group yielded a product with a $C^{\beta}_{22:6}$ content of 10–80 %.

Purified phosphatidyl cholines were prepared from egg yolks (obtained within the previous 24 h) using DAWSON's[9] modifications of previously published procedures[10,11]. Phosphatidyl choline fractions of varying unsaturation were obtained by column chromatography on silicic acid CC4 (Mallinckrodt) at a loading of 1 g crude

TABLE I

EFFECT OF FATTY ACID COMPOSITION OF DIET

Fatty acid composition of two types of diet fed to chickens showing the effect that this had on the composition of crude phosphatidyl choline fractions prepared from eggs laid one month after starting on the diet. Fatty acids present in less than 1 % of the total have not been given unless they were present in higher amounts in other fractions. ND indicates that a particular fatty acid could not be detected.

Fatty acid	*Fatty acid composition (mole %)*			
	Diet		*Egg phosphatidyl choline*	
	Tallow	*Cod/pilchard oil*	*Chickens fed tallow*	*Chickens fed cod/pilchard oil*
$C_{14:0}$	2.6	5.3	ND	ND
$C_{16:0}$	23.3	15.5	23.9	29.8
$C_{16:1}$	ND	10.0	ND	ND
$C_{18:0}$	16.0	ND	17.4	14.2
$C_{18:1}$	39.6	17.8	33.3	28.6
$C_{18:2}$	12.0	7.9	12.3	6.5
$C_{18:3}$	2.3	1.4	1.1	ND
$C_{20:1}$	ND	6.4	0.1	0.6
$C_{20:4}$	ND	0.9	5.0	1.5
$C_{20:5}$	4.2	21.2	0.1	2.9
$C_{22:5}$	ND	1.9	0.4	ND
$C_{22:6}$	ND	12.0	4.4	14.3

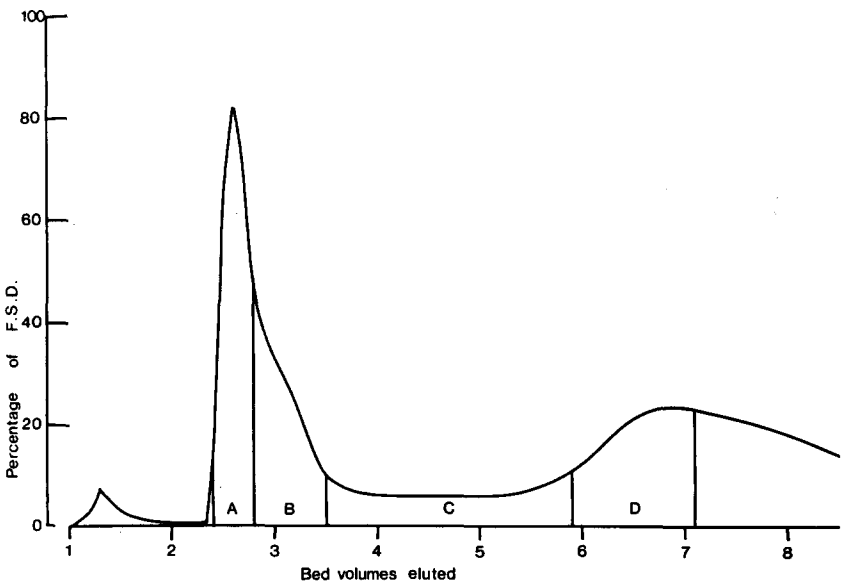

Fig. 1. Silicic acid chromatography of enriched phosphatidyl cholines. Crude egg phosphatidyl cholines, prepared from egg yolks collected from chickens fed on a fish-oil containing diet and partially purified on a column of alumina[9], were chromatographed on a silicic acid column (Mallinckrodt CC_4–1 g phospholipid per 40 g silicic acid) using chloroform–methanol (2:1, v/v). The effluent was monitored on a Pye Liquid Chromatograph with Argon Ionization Detector. The relative amount of phosphatidyl choline in each fraction (based on lipid phosphorus determinations) was: A, 350; B, 475; C, 333; and D, 745. The positional analyses are shown in Table II. F.S.D. = full-scale deflection.

phospholipid per 40 g silicic acid, using chloroform:methanol (2:1, v/v) as solvent. A typical fractionation of crude phosphatidyl choline from 'fish-oil' eggs is shown in Fig. 1. Fraction A contains the highest proportion of polyunsaturated fatty acids. Great care was taken during the purification to prevent oxidation, manipulations of the extracted lipid being carried out under N_2 where possible. When stored under N_2 in the deep freeze, preparations remained usable for up to two weeks. The yield is 10 mmoles of phosphatidyl choline (all fractions) from 12 eggs.

The characterization and criteria of purity for the different phosphatidyl cholines was as follows. All phosphatidyl choline preparations gave a single spot on thin-layer chromatography in chloroform–methanol–7M ammonia (690:270:45, by vol.)[12], when applied in large amounts to the plate and visualised with Rhodamine-6G and a spray for phosphate[13]. In all cases the ninhydrin reaction was negative, and the only base detectable after hydrolysis and paper chromatography was choline[14]. Phosphorus to nitrogen ratios were always between 0.97 and 1.03. Infra-red spectra, obtained as a dried film on a NaCl disc, were identical with that of a known sample of lecithin. Ultraviolet spectra, recorded in absolute ethanol, were used to detect oxidation, any preparation which gave a ratio $A_{233}:A_{215}$ greater than 0.07 (corresponding to about 0.1 % oxidation) being rejected[15].

Positional analyses of the fatty acids using phospholipase A in damp ether[16] were performed on each fraction. Purified phosphatidyl choline (10–20 mg) was dissolved in 5 ml diethyl ether and a further 5 ml diethyl ether was added containing 0.05 ml aqueous $CaCl_2$ solution (5 mM, pH 7.6), and 2–5 mg *Naja naja* phospholipase A (L. Light & Co., Colnbrook, Bucks.). The reaction was left to proceed at room temperature for 6 h, and then overnight at 4°. Lysophosphatidyl choline was removed by centrifugation and washed thoroughly with small quantities of diethyl ether. The ether extracts were pooled prior to the preparation of the methyl esters.

TABLE II

POSITIONAL ANALYSIS OF PHOSPHATIDYL CHOLINE FRACTIONS OBTAINED BY SILICIC ACID CHROMATOGRAPHY OF CRUDE EGG YOLK PHOSPHATIDYL CHOLINES FROM CHICKENS FED ON A DIET CONTAINING FISH OILS

The fatty acid composition was determined by gas–liquid chromatography of the methyl esters. Values less than 1 % of the total are shown only if that fatty acid exceeds 1 % of the total in one or more of the other fractions analysed. A, B, C, and D refer to the different fractions, and are the same as those in Fig. 1. α and β refer to the position on the glycerol skeleton at which the fatty acid is esterified. ND indicates that the particular fatty acid could not be detected.

Fatty acid	*Fatty acid composition (mole %)*							
	A		*B*		*C*		*D*	
	α	β	α	β	α	β	α	β
$C_{16:0}$	55.4	0.9	65.1	0.8	68.2	1.3	70.3	2.1
$C_{18:0}$	36.8	0.4	27.4	0.3	25.7	0.7	25.0	1.5
$C_{18:1}$	5.4	0.9	6.6	15.1	5.3	38.2	4.2	59.4
$C_{18:2}$	0.6	0.8	ND	11.6	ND	17.9	ND	9.1
$C_{18:3}$	1.7	ND	ND	ND	ND	ND	ND	ND
$C_{20:4}$	ND	1.8	ND	3.7	ND	2.9	ND	1.6
$C_{20:5}$	ND	10.6	ND	15.7	ND	8.5	ND	5.0
$C_{22:5}$	ND	5.9	ND	5.1	ND	3.1	ND	1.8
$C_{22:6}$	ND	78.8	ND	46.7	ND	25.7	ND	18.1

Methyl esters were prepared from phosphatidyl choline and lysophosphatidyl choline by transmethylation in methanolic sodium hydroxide[14,17]. Free fatty acids were converted to their methyl esters by reaction with ethereal diazomethane freshly generated from Diazald[18] (Aldrich Chemical Co., Milwaukee, Wisc.). Methyl esters were chromatographed on a Pye series 104 gas chromatograph, and were identified from their retention times and by comparison with known standards[19]. The absolute amount of each component present was calculated from the peak height and retention time using external standards[20]. All compositions are expressed as mole %. The results obtained for the various fractions are shown in Table II.

These results demonstrate that it is possible to obtain phosphatidyl cholines of widely different degrees of unsaturation in quantities of 0.5–1.0 mmole, depending on the number of eggs used. A preparation may be completed within 5 days, and has the advantages of reproducibility, convenience, and relative cheapness.

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Institute of Animal Physiology,
Agricultural Research Council,
Babraham, Cambridge (Great Britain)

R. A. KLEIN

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