jugated *trans*-unsaturated fatty acids from seed oils. Previously known acids were *trans*-5-octadecenoic acid and *trans*-5,*cis*-9,*cis*-12-octadecatrienoic acid from *Thalictrum polycarpum* (5) and *trans*-9,*trans*-12-octadecadienoic acid from *Chilopsis linearis* (7).

The *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid from *C. urticaefolia* seed oil is also a member of an expanding new class of naturally occurring fatty acids containing wide separation of double bonds (1,5,6,8,9,11 and 12).

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Chromatographically Homogeneous Lecithin from Egg Phospholipids[1]

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Abstract

Chromatographically homogeneous egg lecithin, as determined by TLC on Silica Gel G, has been isolated from crude egg phosphatides by column chromatography on alumina through modification of existing, lengthy methods. The modified method involved application of crude egg phosphatides to a column of alumina in the proportion of 1 g phosphatide/25 g alumina, and elution of the lecithin fraction with the 2-component solvent system chloroform:methanol, 9:1 by vol. This method of purification separated lecithin from other choline and non-choline components of crude phosphatides, avoided overloading of the alumina column, and made unnecessary the need for a second chromatographic fractionation of partially purified lecithin on silicic acid, which is needed in existing methods of purification of lecithin.

The use of fresh yolks permitted easier removal of pigment from the final product than was possible with commercially dried yolks.

Phosphatides extracted from dried yolks were much more highly colored than were the phosphatides extracted from fresh yolks and the color presisted through chromatography on alumina.

The fatty acid/phosphorus molar ratio of the purified lecithin was 2.00, which is the theoretical FA/P molar ratio of phosphatidylcholine; other materials with this ratio were not present.

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Introduction

COLUMN CHROMATOGRAPHIC METHODS primarily intended for the analytical fractionation of egg yolk phosphatides have received the attention of several investigators. The initial reports are those of Hanahan et al. (4), and Lea et al. (5). Hanahan et al. used alumina as the adsorbent, and eluted with 95% ethanol to separate choline from non-choline fractions, but were unable to obtain pure phosphatidylcholine free from lysolecithin, as shown by a subsequent report (3). Lea et al. used silicic acid as an adsorbent and eluted pure egg lecithin from a crude egg phospholipid mixture with chloroform:methanol, 8:2 by vol. The latter method is extremely time-consuming. With a more polar solvent system consisting of chloroform:methanol, 1:1 by vol, Rhodes and Lea (10) separated choline and non-choline fractions of egg phospholipids on an alumina column, but had to employ a second fractionation of the choline fraction on a column of silicic acid to remove lysolecithin from lecithin. None of the above-mentioned chromatographic methods were capable of separating pure egg lecithin from a mixture of egg phospholipids on a column of alumina.

Renkonen (9), in an investigation of the breakdown of pure synthetic lecithin on alumina adsorption columns, applied the synthetic lecithin to a column of alumina and successfully separated the pure lecithin from its degradation products (principally lysolecithin) with a solvent system consisting of chloroform:methanol, 9:1 by vol. This ratio of solvents is much less polar than the system employed by Rhodes and

TABLE I

Fractions of Egg Phosphatides Eluted from Alumina with Chloroform: Methanol 9:1 (Fractions 1–8), and with Chloroform:Ethanol:Water 2:5:2 (Fractions 9–14); Applied Phospholipids 25 g

Fraction No.	Fraction vol	Appearance	Fraction wt
	ml		g
1	250	Clear	0
2	250	Clear	0.15
3	90	Hazy	0.28
4	320	Cloudy	12.54
5	273	Hazy	2.47
6	270	Clear	0.63
7	270	Clear	0.44
8	250	Clear	0.17
9	400	Clear	0.12
10	300	Clear	0.75
11	300	Yellow	1.21
12	300	Yellow	4.78
13	300	Clear	0.49
14	300	Clear	0.08

Lea on the same type of adsorbent. Renkonen made no attempt to separate pure lecithin from a mixture of crude phosphatides.

During the course of an investigation in which considerable quantities of chromatographically homogeneous egg phosphatidylcholine were needed, it became desirable to investigate the purification of crude egg phosphatide by means of column chromatography on alumina, for the purpose of developing a relatively rapid procedure which would provide reasonable yields of the pure lecithin. The advantages of employing alumina rather than silicic acid as an adsorbent are: ca. a 5-fold faster flow rate without the necessity of applying pressure, and ca. a 60-fold increase in sample-to-adsorbent ratio. The present report gives the results of an investigation of the extraction of crude egg phosphatides from egg yolks, and a rapid method by which chromatographically homogeneous lecithin was separated from the mixture of phosphatides by column chromatography on alumina.

Experimental

Material and Methods. The egg yolks used in this work were obtained from fresh eggs, and were used either immediately after separation from the whites or after lyophilization and storage at −20C. Dried yolks from a commercial source also were used, as indicated.

All solvents were A.R. grade, used as received.

Aluminum oxide (Merck), "suitable for chromatographic adsorption," was used as received as the adsorbent for column chromatography.

Silica Gel G (Merck), "for thin layer chromatography," was used for TLC analysis. The TLC plates were prepared in the following manner. A paste of the Silica Gel with water, 19 g to 40 ml, was spread to a thickness of 0.5 mm on glass plates. The coated plates were air-dried, and activated at 105–110C for 30 min. Prior to use, the activated plates were stored in a desiccator. Phosphatides samples in chloroform solution were applied to the plates, and chromatographs of the phosphatides were developed by the solvent system chloroform:methanol:water, 65:25:4 by vol, in a saturated chamber (7). Chromatographs of the non-phosphorus containing samples were developed by the solvent system petroleum ether:diethyl ether, 90:10 by vol, (6), also in a saturated chamber. Iodine vapor was used for the non-specific detection of chromatographed materials (11). Ninhydrin and Dragendorff reagents were used for confirmation of amine and choline compounds, respectively. Permanent copies of the various chromatographs were made by a Xerox instrument, and these are reproduced in the several figures of this report.

Phosphorus was determined by the method of Bartlett (1).

Total fatty acids were determined by the method of Lea et al. (5).

Methyl esters of the fatty acids were prepared using BF_3-methanol reagent, and the fatty acid composition was determined by GLC with an IDS Model 20 Chromatograph (Barber Coleman Co.), on a column of Gas Chrom "A" coated with diethyleneglycol succinate.

Fractionation of Egg Phosphatide. The method used for the extraction of egg phospholipids from fresh yolks was a modification of the method described by Hanahan et al. (4). Fresh egg yolks (500 g) from commercial White Leghorn eggs were blended thoroughly with acetone (one liter) at 25C, the mixture allowed to stand for 1 hr, and then filtered. The acetone extract, which contained most of the neutral fat and pigment (10,8), was discarded rather than being combined with later ethanol extracts as in the published method. The solids were washed three times with 200 ml portions of acetone previously cooled to 15C, and then were suspended in one liter 95% ethanol, allowed to stand for 1 hr, and the mixture then filtered. The extraction was repeated with 500 ml 95% ethanol, and the combined ethanol extracts were concn to dryness on a steam bath with nitrogen at reduced pressure. The crude phosphatides then were extracted with two portions of 300 ml each of petroleum ether, the extracts were combined, and reduced in volume to 200 ml. The extract, cooled to room temp, then was poured into one liter of acetone (1:5 ratio) at 15C with rapid stirring, and the mixture was allowed to stand until the supernatant cleared (ca. 1 hr). The acetone phase was decanted, and the precipitated phosphatides were washed with cold acetone. The petroleum ether-acetone step was repeated. The crude phosphatides then were placed into a rotary evaporator for the removal of solvent, and stored at −20C until used. The average yield of extracted crude phosphatides from five extractions was 7.2%, based on wt of fresh egg yolks.

The column chromatographic fractionation of the extracted crude phosphatides was done on a column which measured 4 cm I.D. by 87 cm in length, and which was equipped with a stop-cock and a perforated porcelain disc for support of the alumina adsorbent. A layer of glass wool was placed over the porcelain disc, and Celite filter aid added to a depth of ca. 5 mm to prevent elution of fine alumina particles. Alumina was slurried with chloroform, 625 g to 700 ml, and the slurry was added to the column as one portion. Solvent flow immediately was adjusted to 10 ml/min, and an additional 300 ml chloroform was added to wash the alumina. The bed of adsorbent was about 50 cm in height. The length of the column allowed a driving head of 250 ml solvent to be maintained over the bed of alumina, and this head was sufficient for the flow rate desired.

Twenty-five g extracted crude phosphatides were dissolved in sufficient chloroform to give a 5% solution, and added to the column. The alumina-crude phosphatides ratio of 25:1 was found to be minimum for preventing overloading, and for good resolution of the phosphatide. The phosphatides were washed onto the alumina with an additional 500 ml chloroform, and this solvent was allowed to flow through the column until the solvent level was within ca. 1 cm of the surface of the alumina.

The first solvent system for eluting the phosphatide fractions was chloroform:methanol, 9:1 by vol. Flow

ratio throughout was maintained at 10 ml/min. Data which indicate the progress of a typical chromatographic fractionation of crude egg phosphatides on alumina are given in Table I. The progress of the fractionation was followed by TLC. Approximately 500 ml eluate (fractions 1,2) were clear, and contained a small amt of neutral fat. The next approx 100 ml eluate (fraction 3) was somewhat hazy, and TLC analysis of this fraction indicated the beginning of the elution of lecithin. The next portion of eluate was very cloudy, and ca. 325 ml of this cloudy eluate was collected as fraction 4. Fraction 5 was slightly hazy, and fractions 6,7 and 8 were completely clear, and contained decreasing amt of eluted material. The eluting solvent system was changed after fraction 8 to chloroform:ethanol:water, 2:5:2 by vol, and ca. 2 liters of eluate were collected with this system. Approx 600 ml of the eluate (fractions 11,12) were yellow, and apparently contained most of the pigment of the applied sample.

Thin layer chromatographs of the various fractions listed in Table I were prepared, and are represented in Figure 1. Fraction 2 contained most of the neutral lipid of the applied sample. Fractions 3,4 and 5 contained chromatographically homogeneous egg lecithin, with no indication of any other components. Spots on the chromatograms of these fractions were Dragendorff-positive, ninhydrin-negative. The fractions collected after 5, while still containing lecithin, became increasingly non-homogeneous, and contained lysophosphatides and other phospholipids. Fractions eluated with the chloroform:ethanol:water solvent system contained lysophosphatides, sphingomyelin, cephalin and fatty acids among other components.

Of the lecithin fractions eluted with the chloroform:methanol solvent system, fraction 4 contained most of the phosphatidylcholine of the fractionated phosphatides, and this choline material was eluted as a concentrated band in ca. 0.5 liters of solvent. Fractions 4,5 (Table I) were combined and designated as chromatographically homogeneous. The 2 combined fractions represent a yield of ca. 60% of the phosphatides which were applied to the chromatographic column.

The fatty acid composition of the 2 combined homogeneous lecithin fractions was determined by GLC. The results, compared with those of Tattrie (12) and Hanahan et al. (2), in terms of moles % of acid, were:

Fatty acid	This work	(12)	(2)
Palmitic	37.7	35.7	32.0
Palmitoleic	3.1	Trace	1.0
Stearic	9.2	14.9	16.0
Oleic	32.9	37.0	30.0
Linoleic	17.0	12.4	17.0

The total fatty acid content of the homogeneous lecithin was 69.5%; the total phosphorus content was 3.97%; the FA/P molar ratio was 2.01, which is the theoretical FA/P molar ratio of phosphatidylcholine.

Elution of Phosphatides with Other Solvent Systems. The solvent system chloroform:methanol, 1:1 by vol, was employed to determine its eluting characteristics with crude extracted egg phosphatides on a column of alumina. Fractions eluted with this solvent system were not chromatographically homogeneous, although choline and non-choline materials were separated. The TLC of the major lecithin-containing fraction eluted with this solvent system is represented in Figure 2, and shows the non-homogeneous nature of the fraction. The greater polarity of this solvent system, in comparison with the same solvents at a 9:1 ratio, prevented good resolution of the lecithin fraction.

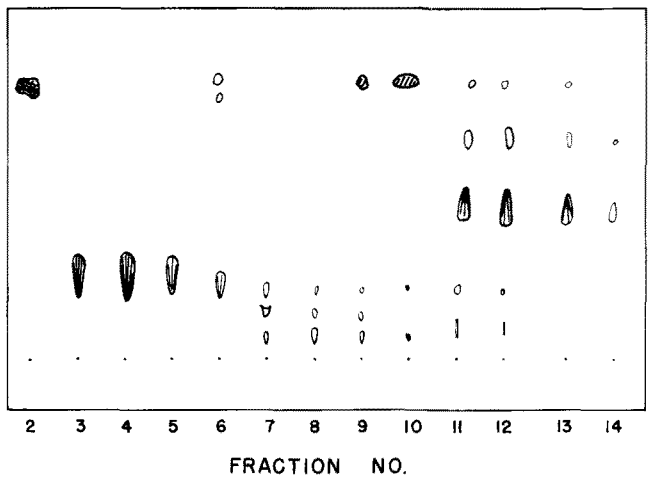

FIG. 1. TLC of fractions of egg phosphatides eluted from a column of alumina with chloroform:methanol, 9:1 by vol (Fractions 1–8), and with chloroform:ethanol:water, 2:5:2 by vol (fractions 9–14).

Homogeneous lecithin was not separated by 95% ethanol as the eluting solvent, as also shown in Figure 2. Choline and non-choline materials were, however, separated.

Extraction of Phosphatides from Fresh Yolks with Various Solvents. Several different solvents were investigated for extracting crude phosphatides from fresh yolks, including lyophilized fresh yolks, as given in Table II. In each case the first extraction solvent was acetone at different temp, and these extracts were discarded since they contained mainly neutral fat. The solid residue which remained after acetone extraction then was extracted with the solvents as shown in Table II, with the respective yields of crude extracted phosphatides as listed. The yield of Run 1 was greatest, due to the fact that water content of the yolks was removed by lyophilization prior to the extraction, and probably also because of incomplete removal of oil by the cold acetone, hence the subsequent extraction of this residual oil, together with the phosphatides. The yields of crude extracted phosphatides of Runs 2 and 3 essentially are equal, and are slightly higher than the yield of Run 4. In the latter run, with ethanol as the extracting solvent, lecithin is preferentially extracted, whereas petroleum ether (Run 2) and chloroform (Run 3) extract the cephalins also, thereby increasing the apparent yield of crude extracted phosphatides in these 2 extractions. The absence of water in the starting material of Run 4 (lyophilized) probably accounts for the yield being higher than the yield of Run 5.

The extraction of lyophilized yolks with solvents was somewhat difficult because this material was somewhat gummy, and was difficult to disperse in added solvent. Since the time consumed by lyophilization is not offset by increased yields of pure lecithin or by other advantages, there seems to be little need for this procedure in obtaining pure lecithin from egg yolks.

The crude extracted phosphatides from each of the extraction procedures given in Table II then were individually fractionated on identical weights of alumina as described, with identical wt of each extracted phosphatide sample applied to the columns, and each was eluted with the chloroform:methanol, 9:1, sol-

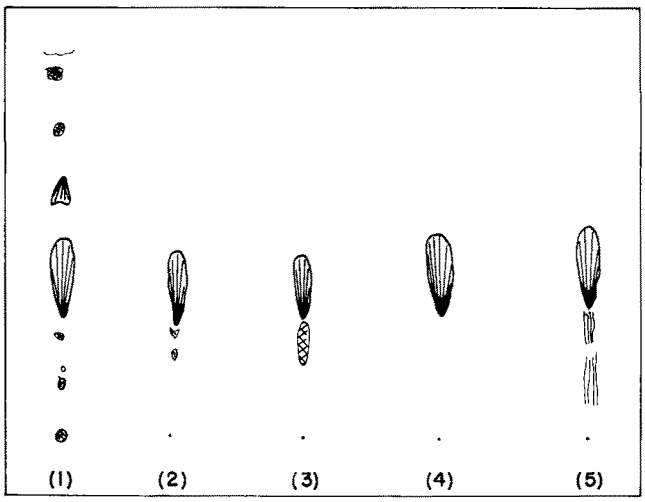

FIG. 2. TLC of various egg phosphatides: 1) crude extracted phosphatides as applied to column of alumina; 2) phosphatide eluted with 95% ethanol; 3) phosphatide eluted with chloroform:methanol, 1:1 by vol; 4) lecithin after heating for 2 hr under nitrogen; and 5) lecithin after exposure to air at room temp for 24 hr.

vent system. The yields of chromatographically homogeneous lecithin obtained by column chromatography, Table II, unquestionably show that the conditions of phosphatide extraction of Run 5 are best. From identical starting wt of crude phosphatide applied to the respective columns, each of which contained the same wt of alumina and was eluted with the same solvent system, the yield of chromatographed lecithin was greatest from extraction Run 5. The conditions of Run 5 most efficiently conserve alumina and time. The overall yields from identical starting wt of fresh yolks are of the same magnitude.

Extraction of Phosphatides from Commercial Dried Egg Yolks. Several attempts were made to extract and chromatograph the phosphatides from commercial dried egg yolks by the same procedures used with fresh egg yolks. The crude phosphatides extracted from dried yolks were much darker than were the phosphatides extracted from fresh yolks. Attempts to remove this dark color by petroleum ether solution—acetone precipitation treatments were not successful. When a chloroform solution of this extracted crude phosphatide was applied to a column of alumina, a yellow pigment was adsorbed immediately on the alumina, and an attempt to elute this pigment from the column preferentially with three liters of chloroform was not successful. The yellow pigment was eluted with chloroform: methanol, 9:1 by vol., in approx the same fractions as the lecithin, which consequently were yellow in color. This yellow pigment band was not observed at any time with fresh yolks, and possibly was due to oxidation products in the dried yolks.

Effect of Heat and Air on Lecithin. The purification of egg phosphatides involves subjecting the material to heat for the removal of solvent, and several transfers from various containers. The effect of heat and air on lecithin was determined by heating a portion of homogeneous lecithin on a steam cone, under nitrogen, for 2 hr, and by allowing another portion of lecithin to stand in an open container for 24 hr at room temp. TLC analyses then were made, and the results are represented in Figure 2. Heating under nitrogen apparently did not result in the formation of degradation products, as this material was chromatographically homogeneous. The sample exposed

TABLE II
Extraction of Egg Phospholipids with Various Solvents, and Yields of Homogeneous Lecithin Obtained by Column Chromatography

Run No.	Temp of initial solvent[a]	Solvent for phosphatide extraction	Crude phos. yield[b]	Chromatographed lecithin yield[c]	Overall yield of lecithin[d]
	C		%	%	%
1[e]	−20	chloroform: methanol 1:1	22.3	18.6	4.1
2	−5	pet. ether	12.7	28.7	3.6
3	−5	chloroform	13.0	31.0	4.0
4[e]	−5	ethanol	11.5	44.8	5.2
5	25	ethanol	7.3	61.2	4.5

[a] Acetone in each instance.
[b] Yield based on wt of yolks.
[c] Yield based on wt of crude phosphatides applied to column.
[d] Yield based on starting wt of yolks.
[e] Lyophilized yolks. all others non-lyophilized.

to air, however, darkened and appeared to contain breakdown products, as determined by TLC. Another sample of lecithin, sealed under nitrogen, was stored at room temp for two weeks without changing color, and remained chromatographically homogeneous.

Lecithin Breakdown on Alumina. Renkonen (9) estimates that ca. 1% lecithin is degraded, substantially to lysolecithin, in 1 hr when in contact with alumina at 22C, and that the production of lysolecithin can be markedly reduced if the lecithin is eluted at 2C. However, in his investigation of lecithin and its degradation, Renkonen used a much more polar solvent system than is represented by chloroform:methanol, 9:1. Since in the present study the latter solvent was used to elute the lecithin fractions, lysolecithin was not detected in the major fractions. Also, one chromatographic fractionation was done at 4C, and the major fraction of this lecithin was identical to that obtained at room temp as determined by TLC.

Discussion

The method presented in this report provides for a rapid, improved and convenient means for obtaining chromatographically homogeneous egg lecithin on a relatively large scale. Although Rhodes and Lea were not able to separate lecithin from lysolecithin and sphingomyelin with the same solvents and alumina, it is felt that this was accomplished in the present work by the solvent ratios employed, and the ratio of sample to adsorbent. These charge and solvent ratios may not be applicable to separating lecithin from other sources, but seem to be very effective for separating the components of crude egg phosphatides. Also, this method may not be applicable for the most efficient yields of phosphatidylethanolamine, since ideally the latter material remains adsorbed on the alumina. However, as a method for purifying egg lecithin, rather than as an analytical method for determination of all of the components of egg phospholipids, the present method is considered to be very satisfactory.

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