J. Lipid Research, October, 1961
Volume 2, Number 4

Evidence for the physiological occurrence of lysolecithin in rat plasma*

H. A. I. Newman, Ching-Tong Liu, and D. B. Zilversmit†

Department of Physiology, University of Tennessee, Memphis 3, Tennessee

[Received for publication March 16, 1961]

SUMMARY

An improved separation of phospholipids on activated silicic-acid columns was achieved by stepwise elution with increasing concentrations of methanol in chloroform. Chemical analyses and paper strip chromatography demonstrated that the noncholine-containing fraction was free of choline-containing phospholipids, and that the lecithin and lysolecithin were of high purity. Rat plasma was shown to contain on the average 17.5% of its lipid phosphorus in the form of lysolecithin. The following is evidence that plasma lysolecithin is not an *in vitro* breakdown product: (*a*) rapid glass-paper chromatography of lipid extracts showed the presence of a distinct spot with the R_f of lysolecithin, (*b*) after P^{32} injection the specific activity of lysolecithin was less than that of lecithin, (*c*) silicic-acid chromatography of C^{14}-labeled plasma lipids failed to show a significant liberation of fatty acids, and (*d*) rechromatography of P^{32}-labeled lecithin did not give rise to a lysolecithin peak.

While lysolecithin has been demonstrated in solvent extracts of human plasma (1 to 5), thus far no evidence has been presented that lysolecithin is present in the blood of intact animals. It would therefore appear legitimate to inquire whether the lysolecithin in lipid extracts might be a degradation product produced during extraction, purification, or chromatographic analysis. In the present paper evidence is presented for the existence of lysolecithin in the blood of intact rats.

Published methods for the separation of plasma phospholipids either failed to detect lysolecithin (6, 7, 8), or the lecithin and lysolecithin (2, 3) fractions were not very pure. Combination of the small silicic-acid column of Phillips (2) with the approximate solvent proportions proposed by Hanahan *et al.* (9) allowed the isolation of these two phospholipids from rat plasma with sufficient purity for our measurements.

METHODS

Normal Holtzman male rats weighing 500 to 600 g were maintained on Purina laboratory chow ad libitum. Large rats were purposely employed so that sufficient plasma phospholipids could be obtained from individual animals for the isotopic studies. In several experiments rats received a single injection of inorganic P^{32} intraperitoneally. At 6, 16, 18, and 20 hours after P^{32} injection, blood samples were taken from the dorsal aorta under Na-pentobarbital (30 mg/kg) anesthesia. Two per cent Mepesulfate (Hoffmann-LaRoche, Inc.) in 0.9% saline was used as an anticoagulant unless otherwise stated. Blood samples were placed in ice water and immediately centrifuged at 1,000 × *g* for 10 minutes at 4°. The supernatant plasma was extracted at 4° overnight with chloroform-methanol 2:1 (v/v)[1] and then purified according to the method of Folch *et al.* (10), except that two gentle

* This work was supported by Grant H-1238 from the National Heart Institute, National Institutes of Health. It was submitted in partial fulfillment of the requirements for the Master's degree of Mr. Liu.

† Career Investigator of the American Heart Association.

[1] The chloroform used in all procedures was freshly distilled technical grade containing 0.25% amylene as preservative. The methanol used in all procedures, except where noted, was freshly distilled technical grade found to contain 3% water.

inversions with equal volumes of distilled water were used for washing. The washed extract was taken to dryness *in vacuo* at room temperature in a rotary flash evaporator and the residue redissolved in 10 ml of chloroform.

Column Separation of Phospholipid Fractions. Four grams of Mallinckrodt 100-mesh silicic acid (suitable for chromatographic analysis), freshly activated[2] by heating at 120° to 130° for 48 hours, was slurried with chloroform and transferred to a 10 × 100-mm column with a 250-ml solvent reservoir. The silicic acid was packed with sufficient pressure from dried air (approximately 1 psi) to maintain a flow rate of 0.5 ml/minute. The phospholipid sample in about 10 ml of chloroform was added, and the pressure increased gradually to 4 to 5 psi to maintain the same flow rate. The eluates from the column were automatically collected through a volumetric siphon in 5-ml fractions.[3] Loads of up to 1.4 mg of phospholipid phosphorus have been employed with satisfactory results.

When the sample had completely entered the column, it was washed with an additional 30 ml of chloroform, which eluted most of the neutral lipids. Approximately 40 to 50 ml of 20% methanol in chloroform (v/v) was then added to the column to elute the noncholine-containing phospholipids. The choline-containing phospholipids, which were left on the column, were separated by adding a series of solvents as follows: 125 to 140 ml of 40% methanol in chloroform (v/v) eluted lecithin; 50 to 80 ml of 60% methanol in chloroform (v/v) eluted sphingomyelin; finally, 50 ml of methanol eluted lysolecithin.

The amount of water in the methanol employed for the eluant mixture was found to be critical. The methanol we employed contained 3% water. The substitution of absolute methanol, prepared by refluxing with magnesium and a trace of iodine, gave poor separations. Distilled technical or reagent grade chloroform could be used interchangeably in the solvent mixture. Other factors that had to be controlled were the particle size and the condition of the silicic acid. Use of the silicic acid prepared by the method of Hirsch and Ahrens (7) (Bio-Rad 325 mesh), either in the form shipped or activated according to our conditions, gave far poorer separations than did the original Mallinckrodt product. Nonactivated Mallinckrodt silicic acid also gave poor separations of phospholipid classes. Furthermore, silicic acid, activated and kept in a desiccator over sulfuric acid, gradually lost its ability to separate phospholipid mixtures.

Aliquots from each 5-ml fraction were analyzed for phosphorus by the method of Bartlett (11), modified as follows: the aliquots were evaporated to dryness in a water bath, and then to each sample was added 0.2 ml of concentrated sulfuric acid. This mixture was digested in a heating block at 210° for 30 minutes. Three to four drops of 30% phosphorus-free hydrogen peroxide (Mallinckrodt) then was added to each tube, and the tubes were again heated at the same temperature for another 30 minutes in order to decompose all of the oxidizing agent. Solvent extracts were mounted and counted on aluminum planchets (12).

Separation of C^{14}-Labeled FFA from Phospholipids. C^{14}-labeled fatty acids were prepared from the liver of a rat which had previously received 200 μc of C^{14}-acetate. Hydrolysis of the extracted lipids was carried out with 0.2 M NaOH in the presence of carbon tetrachloride-methanol-water at 60° for 30 minutes according to Dawson (13). After removal of nonsaponifiable lipids, fatty acids were extracted with petroleum ether (Skellysolve B, b.p. 60°–70°). The residue of C^{14}-fatty acids, after solvent evaporation *in vacuo*, was dissolved in chloroform and added to a chloroform extract of neutral lipids and phospholipids of rat plasma, respectively. Both samples were then placed on a silicic acid-Super-Cel 1:1, (w/w) column (10 × 40 mm). The silicic acid (Mallinckrodt) had been previously equilibrated with saturated $CaCl_2$ solution which provided a relative humidity of 30% at room temperature.[4] Neutral lipids and fatty acids were eluted with 20 ml of chloroform, and phospholipids with 20 ml of methanol. In both samples recovery of C^{14}-fatty acids in the chloroform eluate was better than 90%. It is interesting to note that a petroleum ether extract of phospholipid fatty acids, when evaporated at atmospheric pressure at 60° to 70°, furnished fatty acids that could no longer be recovered quantitatively from the silicic-acid column with chloroform or methanol.

Determination of Phospholipid Glycerol. Glycerol was determined by a modification of the method of Van Handel and Zilversmit (14). Chromotropic acid was prepared by dissolving 110 mg of disodium 4,5-dihydroxy 2,7-naphthalene disulfonate (Eastman) in 10 ml of distilled water. To reduce absorbence of the reagent blank, the solution was filtered through What-

[2] Henceforth, this silicic acid will be designated as activated silicic acid. Fractions collected from this column without a sample load showed the absence of phosphorus and the presence of negligible amounts of glycerol-like material (i.e., formaldehyde after periodate oxidation).

[3] In several instances the separation was monitored by running the eluate through a polyethylene tube (PE 160) wound around a 1B85 Victoreen Geiger tube and measuring the P^{32} radioactivity through a rate meter and recorder.

[4] Henceforth, this silicic acid will be designated as nonactivated silicic acid.

man No. 1 filter paper. To the filtrate was added 90 ml of 24 N H_2SO_4, and the mixture was stored in a dark bottle at 4°.

A sample containing up to 100 μg of lipid phosphorus was dried in an Erlenmeyer flask and resuspended in 1 ml of absolute ethanol. Five milliliters of 2 N HCl was added and the sample refluxed for 48 hours. The entire sample was then shaken with 5 ml of distilled Skellysolve F, b.p. 30°–40°, as in the procedure of Carlson and Wadström (15), and filtered through Whatman No. 31 filter paper. To 0.2 ml of the filtrate was added 0.1 ml of 0.01 M KIO_4. After mixing and a 10-minute waiting period, 0.2 ml of 5% $NaHSO_3$ was added. Then 3 ml of chromotropic acid was added and the contents again mixed and heated at 100° for 30 minutes in the absence of light. After the heating period the sample was cooled, 0.2 ml of 10% aqueous thiourea was added, and the contents again mixed. Thiourea was added to lower the reagent blank according to Frisell and Mackenzie (16). The resulting color was read at 570 mμ against 4 μg glycerol standards. To determine the relative amounts of free and phosphorylated glycerol, we analyzed for phosphate on digested and undigested samples.[5] In one instance the total phosphate of the lecithin hydrolyzate was 9.7 μmoles, whereas the inorganic phosphate was 4.6 μmoles. By difference, the glycerophosphate was therefore 5.1 μmoles or 0.53 of the total phosphate. Upon reaction with periodate, one obtains for every mole of glycerol 2 moles of formaldehyde, and for every mole of α glycerolphosphate only one mole of formaldehyde. To obtain the total μmoles of glycerol of the lecithin hydrolyzate, one multiplies the μmoles of formaldehyde by an appropriate factor, in this instance 0.76 [0.47/2 + 0.53].

Preparation of Lecithin and Lysolecithin Standards. Dog bile was extracted and washed according to the method of Folch *et al.* (10), and then phospholipids were separated from neutral lipids on a silicic-acid column. One gram of nonactivated silicic acid-Super-Cel 1:1 (w/w) was slurried in chloroform and transferred to a 10-ml syringe barrel fitted with a glass wool plug. The sample, in chloroform, containing 0.75 mg of lipid phosphorus, was placed on the column, and the neutral lipids were eluted with 20 ml of chloroform. The phospholipid was eluted with 20 ml of methanol. A small amount of silicic acid in the eluate was removed by centrifugation prior to solvent evaporation at room temperature *in vacuo*. The phospholipid, which by column and paper chromatography was shown to be predominantly lecithin, was taken up in peroxide-free diethyl ether and incubated with lyophilized *Crotalus adamanteus* snake venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) to prepare lysolecithin according to the method of Long and Penny (18). Both lecithin and lysolecithin served as standards for paper chromatography.

Other Methods. Characterization of phospholipids by glass paper chromatography was performed by the methods of Brown *et al.* (19) and Dieckert *et al.* (20). All R_f measurements were made from the origin to the center of the spot. Choline-containing phospholipid spots were detected by the Reinecke salt method, and the detection of amino groups was made by the ninhydrin reaction (21). Ester bonds were determined by the method of Rapport and Alonzo (22), as modified by Hirsch and Ahrens (7), who substituted *n*-butyl alcohol for the ethanol employed as the solvent for the ferric perchlorate. Methyl palmitate (California Corporation for Biochemical Research) was used as a standard. Choline was determined by the method of Wheeldon and Collins (23), but the samples were read at 715 mμ, which increased the sensitivity by 10%. Sphingomyelin was differentiated from other phospholipids by the process of mild alkaline hydrolysis (24). The determination of phosphorus in the water phase was found difficult, however, because it involved the evaporation of large volumes of trichloroacetic acid solution for acid digestion. In addition, the salt which formed during the process of saponification made the solution turbid. To eliminate this defect, the samples were evaporated in Erlenmeyer flasks and digested with 1 ml of concentrated sulfuric acid on a hot plate at 210° for 30 minutes. The volumes of other required reagents were proportionally increased. After the color developed, the sample was transferred to a centrifuge tube, cooled in ice, and extracted by shaking and subsequent centrifugation with known volumes of isobutyl alcohol (25). Finally, a small amount of crystalline sodium chloride was added to accelerate the process of the extraction of color. The isobutanol extract was read at 780 mμ.

RESULTS

Characterization of Phospholipid Fractions. The phospholipid fractions of normal rat plasma were found to be separable into four fractions: noncholine, lecithin, sphingomyelin, and lysolecithin (Fig. 1). Lipid phosphorus eluted from 20 silicic-acid columns

[5] Undigested α- or β-glycerolphosphate were found to give no color with the Fisk SubbaRow reagent. There is the possibility that the estimation of free glycerol from periodate oxidation might be in error due to the presence of β-glycerolphosphate, which does not oxidize to formaldehyde. However, at low pH, as in the conditions of our hydrolysis, the equilibrium strongly favors the α form (17).

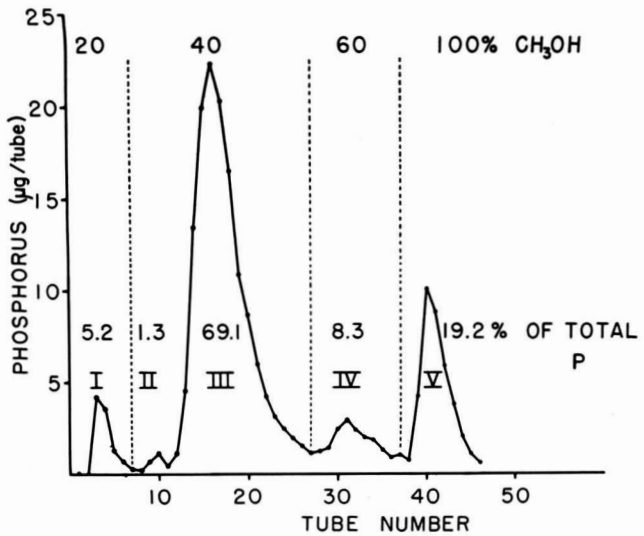


FIG. 1. Representative chromatographic separation of rat plasma phospholipids. The peaks represent: *I* and *II:* noncholine-containing; *III:* lecithin; *IV:* sphingomyelin; *V:* lysolecithin. Phospholipid load: 201 μg P.

was recovered to the extent of 99.1 ± 1.6 S.D. per cent of the amount applied to the column. The amount of lipid phosphorus from each peak was expressed as a percentage of the total lipid phosphorus applied to the column. These percentages were fairly reproducible from sample to sample: in a duplicate run, peak *III* represented 76.5% and 69.9% of the total lipid phosphorus, and in a quadruplicate run, peak *IV* was 8.14%, 8.40%, 7.35%, and 5.40%, while peak *V* was 19.6%, 20.6%, 17.5%, and 19.0% of the total phospholipid phosphorus. There appeared to be little overlap of fractions as evidenced by the characterizations of the five peaks as given below.

Peak I (*Noncholine*). This material containing 5.5 ± 0.2 S.E. per cent[6] of the total lipid phosphorus was eluted with 20% methanol. It was shown to be ninhydrin positive and Reinecke salt negative on paper strip. Its R_f on paper strip is the same as that recorded for phosphatidyle thanolamine and phosphatidyl serine (19).

Peak II. A fraction containing 1.4 ± 0.1 S.E. per cent of the total lipid phosphorus was eluted with 40% methanol. It had insufficient amounts of material to be fully characterized, but preliminary analyses showed it to be all noncholine-containing material.

Peak III (*Lecithin*). The peak *III* material, containing 66.6 ± 1.2 S.E. per cent of the total lipid phosphorus, also was eluted with 40% methanol. In two phospholipid separations the amounts of phosphorus of peak *III* in the aqueous phase obtained after saponification and extraction with chloroform (24) were 98.3% and 97.2% of the total. In a series of five experiments the material in the peak was found to have a mean ester to phosphorus molar ratio of 1.89 ± 0.02 S.E. In one experiment the choline to phosphorus molar ratio was 1.02, and the glycerol to phosphorus was 0.98. As further evidence of identity and purity, the entire lecithin fraction, when subjected to rechromatography, gave only one peak. Moreover, glass paper chromatography gave only one spot with an R_f of 0.77 which corresponded with an R_f of 0.75 for dog bile lecithin (Fig. 2).

Peak IV (*Sphingomyelin*). The peak *IV* material, representing 8.5 ± 0.5 S.E. per cent of the total lipid phosphorus, was eluted with 60% methanol. In two experiments the nonsaponifiable fraction of the eluted material was found to contain 88.3% and 92.6% of the total phosphorus. Furthermore, the R_f on glass paper

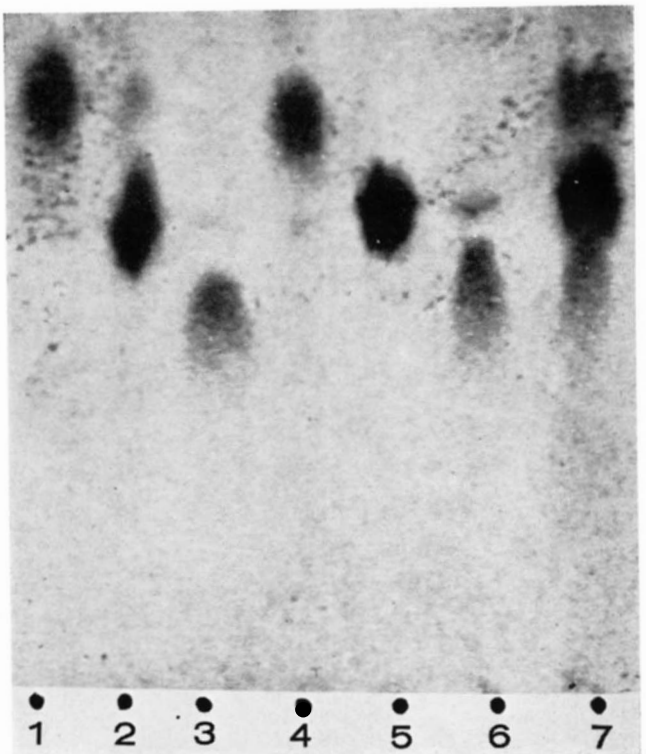


FIG. 2. Silicic acid-impregnated glass paper chromatography of *1*, peak *III*; *2*, peak *IV*; and *3*, peak *V* compared to the standards *4*, lecithin; *5*, sphingomyelin; *6*, lysolecithin; and *7*, a mixture of the three standards. The spots represent 20 μg of phospholipid from the complete pooled peaks derived from four column fractionations. This amount of phospholipid represents an overload in order to detect small quantities of impurities. As little as 0.1 μg phospholipid is detectable by the charring technique. The top of the photograph represents the solvent front. The solvent mixture was phenol-diethyl ether-acetone-water.

[6] All percentages ± S.E. of total phospholipid P are based on a sample of 15 rat plasmas.

was 0.62, and the R_f of sphingomyelin[7] was 0.63. Another spot with the R_f of lecithin was barely discernible (Fig. 2).

Peak V. (*Lysolecithin*). This peak was eluted with pure methanol. This fraction was the second largest and contained 17.5 ± 0.6 S.E. per cent of the total lipid phosphorus. In two experiments the phospholipid was found to be 96.3% and 95.2% saponifiable. In a series of three experiments the ester to phosphorus ratios were 0.95, 1.03, and 0.88. In two experiments the molar ratio of choline to phosphorus was determined and was found to be 0.98 and 1.00, whereas glycerol to phosphorus was 1.11 and 0.99. Paper chromatography showed only one spot with an R_f of 0.49, which corresponded to an R_f of 0.54 for lysolecithin prepared from dog bile lecithin (Fig. 2).

Experiments with Labeled Phospholipids. To test the possibility that lysolecithin eluted with 100% methanol is a breakdown product of lecithin, P^{32}-labeled phospholipids were prepared by injecting two rats intraperitoneally with 200 μc of P^{32}-phosphate each, and obtaining blood 18 hours later. Plasma lipids were extracted and phospholipids separated as described previously. The entire 40% methanol fraction, which in these instances showed only one peak, was evaporated *in vacuo*, extracted in chloroform, and rechromatographed as before. The entire P^{32} load appeared in the 40% methanol fraction with no discernible radioactivity in the 100% methanol eluate. The same type of experiment was performed with a sample of dog bile phospholipids, which is known to contain primarily lecithin. The first chromatographic separation of dog bile phospholipids revealed less than 2% of the total lipid phosphorus as lysolecithin phosphorus. Rechromatography of the lecithin fraction gave rise to less than 1% of the total phosphorus in the 100% methanol eluate. These experiments indicate that a lecithin fraction prepared without prior chromatography (dog bile) yields practically no lysolecithin when subjected to silicic-acid separations. In addition, when chromatographed dog bile or rat plasma lecithin fractions were taken to dryness, re-extracted, and again chromatographed, no breakdown of lecithin was observed. Apparently the procedures involved in the separation of phospholipids as described here are not sufficiently harsh to produce lysolecithin from lecithin as an artifact.

Another way of establishing the independent origin of plasma lysolecithin would be the determination of the specific activities of the lecithin and lysolecithin after P^{32} administration. Table 1 shows these results. One animal killed 6 hours after administration of 100 μc of P^{32}-phosphate showed a lysolecithin specific activity only slightly lower than that of lecithin. Since, according to these data, lysolecithin could have been derived from *in vitro* hydrolysis of lecithin, the time interval after P^{32} administration was prolonged until at 18 to 20 hours all animals showed a considerable difference between the lecithin and lysolecithin specific activities. For all animals the difference was significant at $p < 0.001$. If lysolecithin were a simple *in vitro* breakdown product of lecithin, the specific activities of the two compounds should have been the same unless there were a selective breakdown of part of the lecithin with a specific activity lower than that represented by the whole lecithin fraction.

TABLE 1. SPECIFIC ACTIVITIES OF INDIVIDUAL PHOSPHOLIPIDS OF RAT PLASMA AT DIFFERENT TIME INTERVALS AFTER INJECTION OF P^{32}

Time Interval	Specific Activity (cpm/μg P)				Difference*
	Non Choline-Containing	Lecithin	Sphingomyelin	Lysolecithin	
hours					*per cent*
6	37.0	67.0	34.4	62.4	6.9
16	42.1	77.5	60.3	73.5	5.2
18	48.7	70.0	56.5	52.7	24.7
18	51.9	94.0	68.0	84.9	9.7
18	37.4	76.3	48.2	58.8	23.0
18	19.0	37.5	21.0	19.0	49.4
20	21.0	37.2	28.7	31.2	16.1

* $\frac{(\text{Specific activity of lecithin} - \text{specific activity of lysolecithin}) \times 100}{\text{specific activity of lecithin}}$

That inhomogeneity in P^{32}-labeled lecithin does occur has been shown by Collins (26), who found that upon countercurrent separation, the lecithin of rat liver 90 minutes after injection of P^{32} could be split into three fractions with differing specific activities. Similarly, Harris *et al.* (27) found that P^{32}-labeled rat liver lecithins in successive fractions from silicic-acid columns differed considerably in their specific activities and their fatty acid composition. To examine whether this heterogeneity is present in plasma, one rat was injected with P^{32}-phosphate, and 18 hours later blood was obtained for extraction and silicic-acid chromatography in the usual manner. Each 5-ml eluate was analyzed for P^{32} and P^{31}. The specific activities of the

[7] Kindly supplied by Dr. H. E. Carter.

lecithin fractions are shown in Figure 3. Apparently, as was observed in liver (26, 27), marked differences in the plasma lecithin specific activities exist. In the same plasma the lysolecithin fraction also showed differing specific activities which overlapped in part with those of lecithin. These data therefore cannot exclude the possibility that the lysolecithin was derived from the selective breakdown of a low specific activity lecithin.

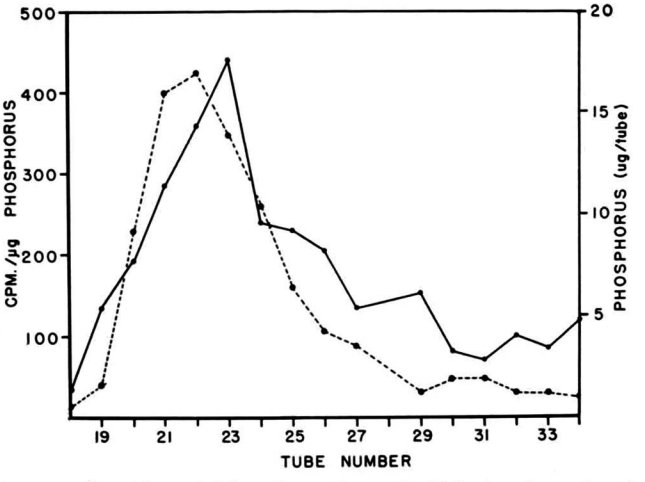


FIG. 3. Specific activities of rat plasma lecithin fractions eluted from silicic acid with 40% methanol. Broken line = phosphorus curve; unbroken line = specific activity curve.

Even if selective breakdown of P^{32}-lecithin on the silicic-acid column is assumed, such breakdown must be extremely rapid, as shown by the following experiment: Rat plasma lipids were extracted with chloroform-methanol 2:1 (v/v) for 5 minutes and concentrated *in vacuo*. Extraction and concentration were performed at 4°. A 5-µl portion of the concentrated lipids was spotted on silicic acid-impregnated glass paper and dried at room temperature for 1 minute. The paper was developed with diethyl ether-phenol-acetone-water within 20 minutes (19). Figure 4 shows a clear lysolecithin spot. Thus, if silicic acid were responsible for the breakdown of lecithin, this would have had to happen very early in the separation, since otherwise streaking of the spot would occur during chromatography.

Another way to test whether lecithin is converted to lysolecithin on the silicic-acid column would be to obtain a plasma phospholipid sample from which the free fatty acids had been removed. Such a sample, when subjected to column chromatography, should furnish free fatty acids only if lecithin breakdown occurred. Therefore 200 µc of acetate-1-C^{14} and 100 µc of P^{32}-phosphate[8] were injected intraperitoneally into a rat 6 hours prior to sacrifice. Plasma lipids were extracted at 4° and the neutral lipids dialyzed through a rubber membrane (28) as shown in Figure 5. The nondialyzable (phospholipid) fraction was found to be free of fatty acids by the glass paper chromatographic technique (29) utilizing 0.5% methanol in petroleum ether (b.p. 60°–70°, Skellysolve B). After chromatography of these phospholipids through the activated silicic-acid column, the chloroform eluate contained 56 cpm (Fig. 5). This could represent a slight contamination of the nondialyzable fraction by neutral lipids or a minimal liberation of fatty acids from phospholipids upon initial

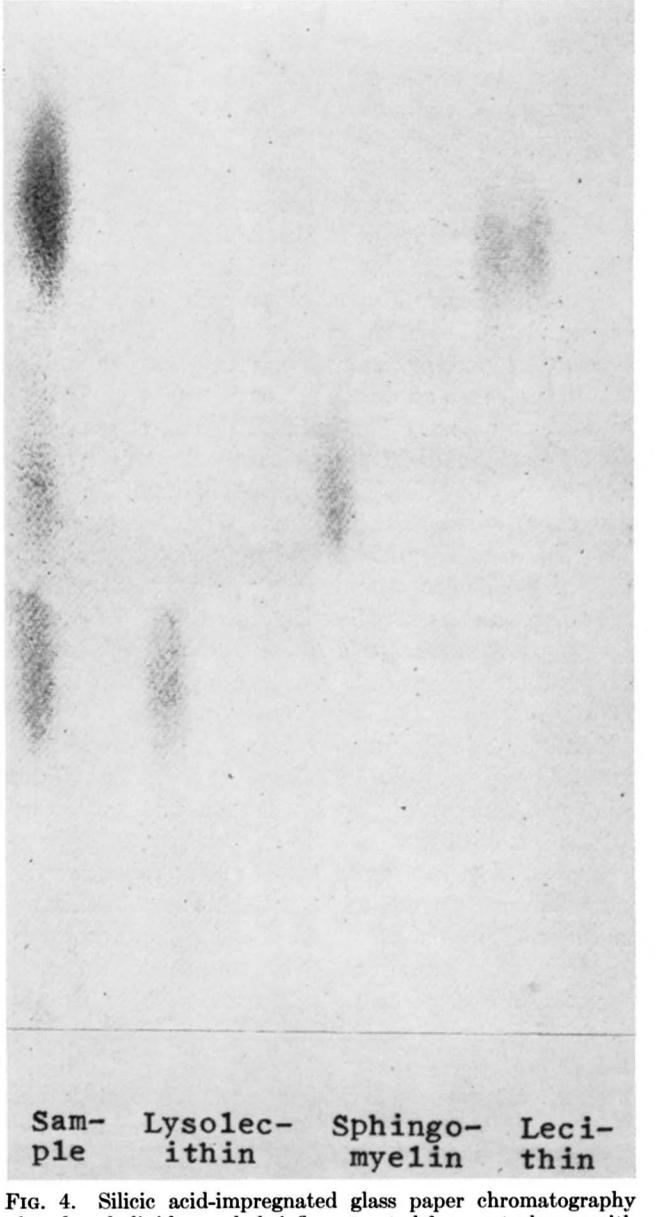
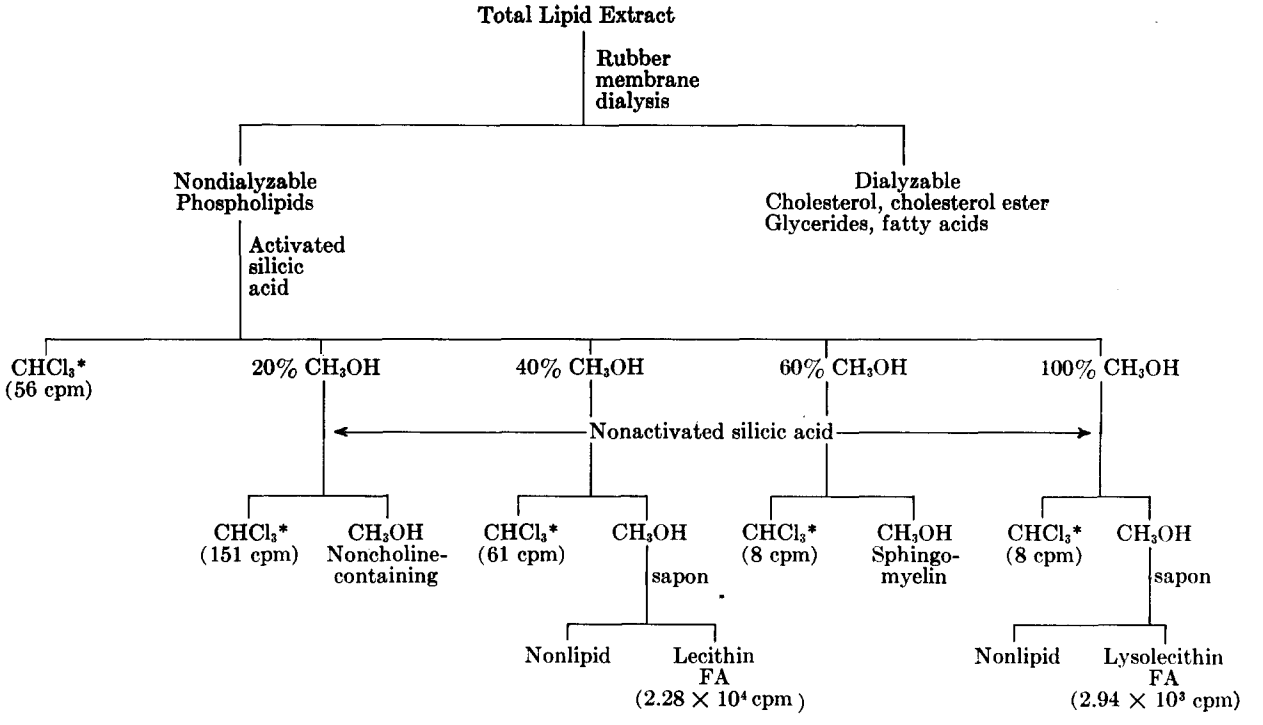


FIG. 4. Silicic acid-impregnated glass paper chromatography of a phospholipid sample briefly extracted from rat plasma with chloroform-methanol and standards of lysolecithin, sphingomyelin, and lecithin.

[8] The P^{32} merely served to monitor the eluates; see footnote 3.

FIG. 5. FLOWSHEET FOR COMBINED DIALYSIS-CHROMATOGRAPHY EXPERIMENT.



* Total C^{14} in chloroform eluates: 284 cpm.

contact with silicic acid. The 20%, 40%, 60%, and 100% methanol eluates were dried separately *in vacuo*, dissolved in chloroform, and rechromatographed with chloroform on nonactivated silicic acid-Super-Cel. The chloroform eluates, which would contain the fatty acids if any had been previously liberated on activated silicic acid, gave a total radioactivity of 228 cpm. The total C^{14} in the lecithin fatty acids liberated by hydrolysis (13) was 2.28×10^4 cpm. In the lysolecithin fatty acids 2.94×10^3 cpm were found. However, in the combined chloroform eluates only 284 cpm appeared, which could represent breakdown of lecithin to lysolecithin. If the specific activities of the α and β fatty acids of lecithin are assumed to be of the same order of magnitude, the C^{14} in the chloroform eluates would at most represent only a small amount of free fatty acids and hence account for only a small portion of the lysolecithin found in rat plasma.

DISCUSSION

Our characterization of the phospholipid fractions separated from rat plasma is indicative of the high purity of lecithin and lysolecithin, although the sphingomyelin was only 90% pure. The Phillips column (2, 3) was tried in our laboratory, but it separated lecithin and sphingomyelin poorly: the lecithin fraction contained considerable quantities of nonsaponifiable phospholipid, presumably sphingomyelin, and the sphingomyelin was also contaminated with lecithin. Furthermore, Phillips found that his lysolecithin was only 79% pure, the contaminants being about 10% sphingomyelin and lecithin each.

The proof of purity of lecithin and lysolecithin obtained from our column depends not only on the chemical characterizations but is also evidenced by rechromatography of these fractions on silicic acid-impregnated glass paper by a solvent system that differed from the one used in the column chromatography. Even overloading the paper by a factor of ten (Fig. 2) failed to show impurities in these two fractions.

The finding of plasma lysolecithin in rat plasma parallels the results of Phillips (2, 3) and Gjone *et al.* (1) in human plasma. However, Hanahan *et al.* (8) reported analyses of human plasma phospholipids in which no lysolecithin was found. This investigator suggested that his failure to find lysolecithin might have resulted from differences in extraction techniques or the use of a different anticoagulant. For this reason a human blood sample was treated with ACD[9]

[9] ACD: Abbott Laboratories.

and extracted with either chloroform-methanol 2:1 (v/v) or ethanol-diethyl ether 3:1 (v/v), the solvent system used by Hanahan. The fractionation on our activated silicic acid gave lysolecithin contents of 10.8% of the lipid phosphorus in the chloroform-methanol extract, and 8.4% of lipid phosphorus in the ethanol-diethyl ether. The lysolecithin content of a rat plasma sample extracted by ethanol-diethyl ether was also about the same as that observed previously in other samples after chloroform-methanol extraction. Another factor which could affect the hydrolysis of a lysolecithin precursor is temperature, but separation of rat plasma by centrifugation for 10 minutes at 4° or room temperature, and subsequent extraction of rat plasma lipids at either of these temperatures, gave the same lysolecithin content. Therefore it seems highly unlikely that lysolecithin could have been formed as an artifact in the interval between removal of blood from the rat and the extraction of lipids from plasma.

The presence of lysolecithin in various biological materials has been asserted by different authors. However, in view of the known hemolytic activity of lysolecithin, its presence in blood in appreciable quantities has been doubted. Neither Hanahan *et al.* (8) nor Nelson and Freeman (6) were able to discover lysolecithin in human blood serum. Douste-Blazy (30), however, found an appreciable quantity of lysolecithin in one sample of human plasma phospholipids studied by countercurrent distribution. Similarly, Hajdu *et al.* (31) reported the existence of lysolecithin in human plasma. Phillips (2, 3) and Gjone *et al.* (1) demonstrated the presence of lysolecithin in human serum by silicic-acid column chromatography, and Marinetti *et al.* (4, 5) confirmed this finding by the application of paper chromatography. Ayrault-Jarrier (32) found lysolecithin in the low density lipoprotein of human serum. Phillips (33), moreover, compared various serum lipoproteins and found relatively more lysolecithin in the high density lipoprotein fraction ($d > 1.063$) than in the low density fraction ($d < 1.063$), while the highest lysolecithin relative to other phospholipids occurred in the protein fraction of density higher than 1.21 (34).

One might ask whether possibly the decomposition of plasmalogens, which are known to be prone to breakdown, could account for the presence of lysolecithin. To study this possibility, a rat serum sample was analyzed immediately after extraction for plasmalogen by the method of Leupold and Büttner (35). Less then 3% of the plasma lipid phosphorus could be found as plasmalogen, and therefore cannot account to even a small extent for the presence of lysolecithin.

The clear separation of lysolecithin from briefly extracted rat plasma by paper chromatography speaks against the concept that lysolecithin is an *in vitro* breakdown product of lecithin. Furthermore, the absence of a lysolecithin peak when lecithin was rechromatographed, and the insignificant liberation of C^{14}-fatty acids from rat plasma phospholipids during silicic-acid chromatography, do not support the hypothesis that lecithin breaks down to lysolecithin during column chromatography. Finally, the difference in specific activities of serum lysolecithin and lecithin would be evidence against a random breakdown of lecithin to lysolecithin *in vitro*. It thus appears almost certain that lysolecithin is a normal constituent of rat plasma.

The authors wish to acknowledge the technical assistance of Mrs. John K. Wadley, Mrs. Jo Northrop, and Mr. Napoleon Williams.

REFERENCES

1. Gjone, E., J. F. Berry and D. A. Turner. *J. Lipid Research* **1:** 66, 1959.
2. Phillips, G. B. *Proc. Natl. Acad. Sci. U.S.* **43:** 566, 1957.
3. Phillips, G. B. *Biochim. et Biophys. Acta* **29:** 594, 1958.
4. Marinetti, G. V., M. Albrecht, T. Ford, and E. Stotz. *Biochim. et Biophys. Acta* **36:** 4, 1959.
5. Marinetti, G. V., and E. Stotz. *Biochim. et Biophys. Acta* **37:** 571, 1960.
6. Nelson, G. J., and N. K. Freeman. *J. Biol. Chem.* **234:** 1375, 1959.
7. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
8. Hanahan, D. J., R. M. Watts and D. Pappajohn. *J. Lipid Research* **1:** 421, 1960.
9. Hanahan, D. J., J. C. Dittmer and E. Warashina. *J. Biol. Chem.* **228:** 685, 1957.
10. Folch, J., M. Lees and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
11. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
12. McKay, B. P., and D. B. Zilversmit. *Nucleonics* **11:** 58, 1953.
13. Dawson, R. M. C. *Biochem. J.* **57:** 237, 1954.
14. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50:** 152, 1957.
15. Carlson, L. A., and L. B. Wadström. *Clin. Chim. Acta* **4:** 197, 1959.
16. Frisell, W. R., and C. G. Mackenzie. In *Methods of Biochemical Analysis*, edited by David Glick, New York, Interscience Publishers, Inc., 1958, vol. 6, p. 70.
17. Burmaster, C. F. *J. Biol. Chem.* **164:** 233, 1946.
18. Long, C., and I. F. Penny. *Biochem. J.* **65:** 382, 1957.
19. Brown, M., D. A. Yeadon, L. A. Goldblatt, and J. W. Dieckert. *Anal. Chem.* **29:** 30, 1957.
20. Dieckert, J. W., W. B. Carney, R. L. Ory, and N. J. Morris. *Anal. Chem.* **30:** 1442, 1958.
21. Hack, M. H. *Biochem. J.* **54:** 602, 1953.
22. Rapport, M. M., and N. Alonzo. *J. Biol. Chem.* **217:** 193, 1955.

23. Wheeldon, L. W., and F. D. Collins. *Biochem. J.* **70:** 43, 1958.
24. McCandless, E., and D. B. Zilversmit. *Arch. Biochem. Biophys.* **62:** 402, 1956.
25. Berenblum, I., and E. Chain. *Biochem. J.* **32:** 295, 1938.
26. Collins, F. D. *Nature* **186:** 366, 1960.
27. Harris, P. M., D. S. Robinson and G. Getz. *Nature* **188:** 742, 1960.
28. Van Beers, G. J., H. de Iongh and J. Boldingh. In *Essential Fatty Acids*, edited by H. M. Sinclair, New York, Academic Press, Inc.; London, Butterworths Scientific Publications, 1958, p. 43.
29. Newman, H. A. I., E. L. McCandless and D. B. Zilversmit. *J. Biol. Chem.* **236:** 1264, 1961.
30. Douste-Blazy, L. *Compt. rend.* **239:** 460, 1954.
31. Hajdu, S., H. Weiss and E. Titus. *J. Pharmacol. Exptl. Therap.* **120:** 99, 1957.
32. Ayrault-Jarrier, M. Thesis, University of Paris, 1959.
33. Phillips, G. B. *J. Clin. Invest.* **38:** 489, 1959.
34. Phillips, G. B. *Proc. Soc. Exptl. Biol. Med.* **100:** 19, 1959.
35. Leupold, F., and H. Büttner. *Z. physiol. Chem., Hoppe-Seyler's* **292:** 13, 1953.