TRANSESTERIFICATION OF LECITHIN ON SILICIC ACID COLUMNS*

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Since silicic acid chromatography is the most commonly used method for the separation and preparation of complex lipids, the nature of chemical changes occurring during this process is a problem of considerable interest. Evidence has been published[1–7] indicating that such changes do occur with phospholipids and glycerides, but since the conditions used by various investigators are appreciably different, there is little agreement on the extent and nature of the changes. During a study of the lipids occurring in mammalian tissues, it was observed that lysolecithin was apparently produced during fractionation on silicic acid. Preliminary experiments indicated that the amount formed was dependent on the length of the column, flow rate of solvent, source of adsorbent, and temperature. Some of these factors were examined more carefully and represent the subject of this report.

MATERIALS AND METHODS

Solvents

All solvents were redistilled.

Adsorbents

Three commercially available preparations of silicic acid were used: "Unisil", 200 to 325 mesh, from Clarkson Chemical Co.; "Suitable for Chromatography", 100 mesh, from Mallinckrodt Chemical Co.; and "Analyzed Reagent", from J. T. Baker Chemical Co., screened and washed as described by RAPPORT *et al.*[9].

Analytical methods

Thin-layer chromatograms were prepared with silica gel G (Merck) containing calcium sulfate binder, using a Desaga spreader[10]. For development two solvent mixtures were used: chloroform–methanol–water (75:25:4) and petroleum ether (30°)–diethyl ether–glacial acetic acid (90:10:1). Lipids were detected by means of either iodine vapor, or charring by heating after spraying with 2 N H_2SO_4. Phosphorus was determined by a modification of the method of BEVERIDGE AND JOHNSON[11], and ester groups by the method of RAPPORT AND ALONZO[12].

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Extraction of tissue lipids

Rat and mouse tissues were extracted by homogenization with 20 volumes of chloroform–methanol (2:1) in a Potter-Elvejem glass homogenizer. Human testis was homogenized in a Virtis "45" homogenizer. After gravity filtration (S. & S. Sharkskin), the lipids were washed with water according to FOLCH *et al.*[13]

Phospholipids

Azolectin (American Lecithin Co.) was found to be a suitable soybean phospholipid fraction. Egg lecithin was obtained from Sylvana Chemical Co. and was free of phosphatidal choline (plasmalogen). Synthetic dipalmitoyl lecithin and dipalmitoyl phosphatidyl ethanolamine were purchased from California Corp. for Biochemical Research. Beef heart lecithin (a mixture of phosphatidyl and phosphatidal cholines) was prepared according to PANGBORN[14]. Phosphatidal choline was isolated by the method of GOTTFRIED AND RAPPORT[15].

Gas–liquid chromatography

Fatty acids were methylated as described by KATES[16] and analyzed with a 6 ft. column of 16.1 % ethylene glycol succinate on 80 to 100 mesh chromosorb W, temperature 171°, Argon gas carrier, and a flame ionization detector.

RESULTS

Changes detected in total lipid extracts and purified fractions

In these experiments crude lipid mixtures or purified preparations containing 500 to 750 μg of phosphorus were chromatographed at 5° on a column of silicic acid measuring 12.5 × 0.8 cm (2.5 g of adsorbent). A discontinuous gradient elution was used in which three fractions were collected: fraction I, chloroform, 50 ml; fraction II, ethanol, 60 ml; and fraction III, methanol, 60 ml. Flow rates were maintained constant by a small positive pressure of nitrogen. The respective solvents were then removed under reduced pressure; the residues were dissolved in chloroform and examined by thin layer chromatography. Using the chloroform–methanol–water development mixture, a large spot migrating like lysolecithin was detected in the methanol eluate of all lipid extracts from rat, mouse, and human testis, and mouse and rat liver. The same was true with egg lecithin and beef heart lecithin. No lysolecithin-like spot was found in any of the unchromatographed mixtures. No changes were detected after chromatography in either phosphatidal choline or a beef heart cephalin preparation (a mixture of phosphatidyl and phosphatidal ethanolamines). No changes in any of the fractions were detected as a result of any chemical manipulations if contact with the adsorbent did not occur.

The lysolecithin was characterized after elution from preparative thin layer plates by the ratio of ester groups to phosphorus, for which the value found was 1.00. In all methanol eluates in which lysolecithin was found, thin layer chromatograms also showed the presence of another substance which migrated very rapidly and was therefore of low polarity. This material was clearly a second reaction product of the column, since none was found in the previous two eluates in which less polar lipids normally appear. In the case of the purified lipids, no nonpolar lipid fraction was detectable before column chromatography. Development of thin layer chromato-

grams with petroleum ether–ethyl ether–acetic acid showed that most of the non-polar fraction migrated similarly to fatty acid methyl esters, and lesser amounts behaved like free fatty acids. Their identity was confirmed by gas–liquid chromatography after elution from preparative plates. The ester fraction was subjected to gas–liquid chromatography directly and the free acids were first esterified. These experiments showed that the lecithin eluted from silicic acid with methanol participated in a transesterification reaction with the methanol, in which the major products were lysolecithin and methyl esters of fatty acid. The failure of phosphatidal choline to react indicates that this transesterification reaction is probably limited to fatty acids bound to primary alcoholic groups.

Effect of flow rate on extent of reaction

The dependence of the reaction on flow rate of methanol was studied as follows:

10 mg of egg lecithin was loaded on to silicic acid columns containing 2.5 g of adsorbent. Two fractions were collected, the first with 50 ml of absolute ethanol, and the second with 60 ml of methanol. All the lipid was found in the second fraction. The methanol was allowed to pass through each column at a constant rate, but the rate was changed in each experiment. After removal of the solvent under reduced pressure, the residue was dissolved in 5 ml of chloroform. Analysis of this residual lipid for methyl esters was effected by placing portions of the chloroform solution on microcolumns of silicic acid prepared with 0.4 g of "Unisil" ("Unisil" was found to be inert for methyl esters). Eluates obtained with 20 ml of chloroform contained all the methyl esters and free fatty acids, and these were then measured.

To determine the extent of conversion of lecithin to lysolecithin, a portion of the methanolic fraction from the reaction column was developed on a thin layer chromatogram with chloroform–methanol–water, the spots were made visible with

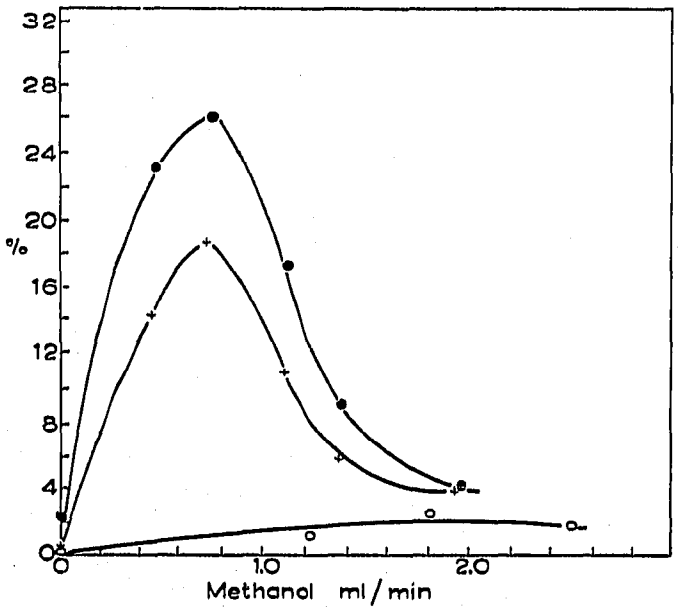

Fig. 1. Effect of flow rate of the eluting solvent. ● = % of lecithin phosphorus converted to lysolecithin phosphorus, Baker's silicic acid column; + = % of lecithin acyl esters converted to fatty acid methyl esters, Baker's silicic acid column; O = % of lecithin acyl esters converted to fatty acid methyl esters, "Unisil" silicic acid.

iodine vapor, and those corresponding to lecithin and lysolecithin were scraped off and analyzed for phosphorus. A corresponding area from an unused "lane" was used as a control.

There was a marked dependence of the extent of reaction (lecithin + MeOH = lysolecithin + fatty acid methyl ester) on the flow rate of methanol (Fig. 1), when the adsorbent was Baker's silicic acid. With "Unisil" the effect was less striking. With Mallinckrodt silicic acid, the effect was more pronounced than with "Unisil", but the fineness of the particles made it difficult to obtain comparable rates of flow.

The experiments shown in Fig. 1 were run at 5°. With columns run at room temperature (26°) the extent of reaction using egg lecithin was about 50 % *less* at comparable rates of flow.

Effect of prolonged contact

To evaluate the effect of prolonging the contact of lecithin with silicic acid in a stationary methanol phase, the following experiment was carried out. Lecithin (5 mg) in an appropriate volume of solvent was loaded on a column containing 0.5 g of silicic acid. Solvent flow was prevented by means of a side arm siphon device. After varying lengths of time (0, 2, 4, 16, 48 h), the lipid was eluted rapidly by adding 20 ml of ethanol followed by 30 ml of methanol. Lysolecithin and fatty acid methyl esters were determined on the methanol eluate using the procedure described. All of the

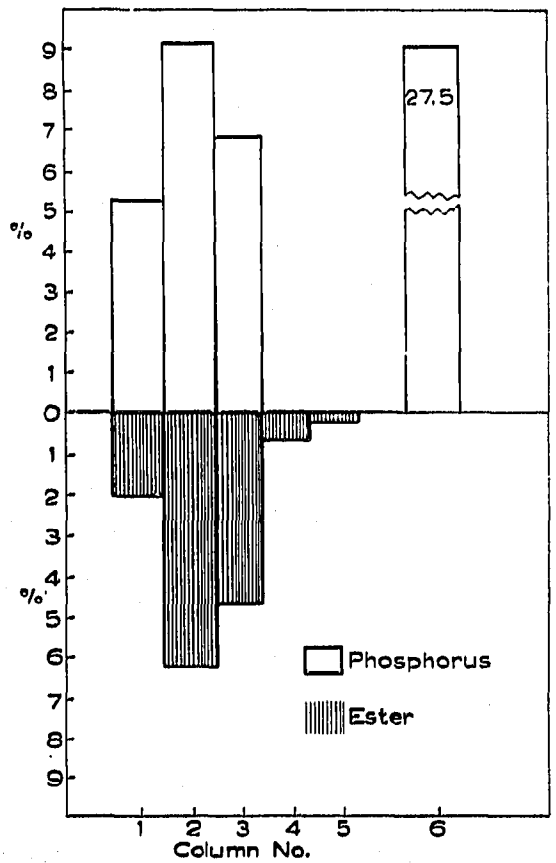

Fig. 2. Effect of product removal with sequential columns. □ = % of lecithin phosphorus converted to lysolecithin phosphorus on each column. ▥ = % of lecithin acyl esters converted to fatty acid methyl esters on each column.

lipid placed on the column was recovered. At zero time, no lysolecithin was formed with any of the 3 silicic acid preparations. After 2 h contact, only 0.5 to 2.0 % of the lecithin was converted to lysolecithin and no increase was found after the longer periods, including that of 48 h. The columns prepared with Baker's silicic acid resulted in greater conversion (2 %) than those with Mallinckrodt's silicic acid or "Unisil". A higher degree of conversion was also found more frequently at 5° than at 26°.

Effect of product removal

Six similar columns of Baker's silicic acid measuring 12.5 by 0.8 cm were prepared. Egg lecithin (20 mg) was placed on the first columns and two fractions were collected, one with 50 ml of chloroform, and the second with 60 ml of methanol. The methanolic eluate contained lysolecithin, fatty acid methyl esters, and unchanged lecithin. The solvent was removed, and the residue was dissolved in chloroform. A small portion was removed for analysis, and the remainder was placed on the second column. The procedure was repeated with the rest of the six columns and thus permitted the removal (in the chloroform eluate) of fatty acids and fatty acid methyl esters produced at each step, whereas the lysolecithin continued to accumulate.

To test the effect of removal of lysolecithin, the methanolic eluate from column 5 was separated on a preparative thin layer chromatogram, and the unchanged lecithin fraction, after elution, was placed on the sixth column. From the results of these experiments, shown in Fig. 2, it is evident that lecithin breakdown occurred only on the first three columns, and no further conversion to lysolecithin was detected on columns 4 and 5. However, after removal of the lysolecithin product, further conversion occurred (column 6). Gas–liquid chromatography of the fatty acid esters formed on columns 1, 2, 3 and 6 indicate some differences in the lecithin molecules that reacted on each column (Table I).

TABLE I

METHYL ESTERS FORMED DURING REPEATED PASSAGE OF EGG LECITHIN THROUGH SIMILAR SILICIC ACID COLUMNS

Major fatty acid component	*Relative percentage*			
	Column No. 1	*Column No. 2*	*Column No. 3*	*Column No. 6*
16:0	26.3	28.9	42.3	31.8
16:1	—	—	—	10.4
18:0	21.6	19.8	16.5	16.8
18:1	37.8	31.2	25.6	33.3
18:2	14.3	20.0	15.6	7.7
Ratio saturated: unsaturated	0.91	0.95	1.43	0.95

DISCUSSION

Chromatography on silicic acid has up to now been the method of choice for lipid fractionation. However, there are indications that it is not an ideally inert adsorbent. MERCKL AND LANDS[5] observed the migration of fatty acyl groups of

lysolecithins when passed through silicic acid columns. LEA *et al.*[4] claimed that the lysolecithin detected after the fractionation of egg-yolk phospholipids could be a degradation product occurring during the extraction and separation on silicic acid. On the other hand, RENKONEN[7] AND NEWMAN *et al.*[25] presented results indicating that lecithins were not degraded by this type of column chromatography. I have found that lecithins are partially degraded on silicic acid columns. The nature of the products formed points to a general reaction that could be written as:

$$\text{Lecithin} + \text{MeOH} + H_2O = \text{lysolecithin} + \text{RCOOMe} + \text{RCOOH}$$

The reaction is shifted towards the formation of lysolecithin by the movement of the solvent (methanol) through the columns, the lysophosphatide was only formed when methanol was used as the eluant. Hexane, chloroform, ethanol and ethanol–water mixtures did not lead to lecithin breakdown. The efficiency of methanol in driving the reaction towards transesterification and hydrolysis can be explained by the polarity of this solvent and a mass-action effect. Methyl esters, fatty acids and lysolecithin are separated from each other and from the parent lecithin as soon as formed, since the rates of elution from silicic acid columns are different for each one of these compounds. The mass-action effect is accelerated by increased flow rates of eluting solvent, up to a point where the period of contact between the reactants and the silicic acid acting as catalyst becomes limiting. When product removal is achieved by the more efficient device of sequential columns, as much as 70 % of lecithin can be converted to lysolecithin.

The amount of lysolecithin formed was dependent upon the commercial silicic acid preparation used. This might indicate that the catalyst is not the silicic acid itself but a contaminant present in different quantity in each preparation. WALLING[17] showed the presence of appreciable quantities of sulfuric acid in several commercial preparations of silicic acid. However, no excess acid or base was detectable in the washed adsorbents used here. An alternative explanation is that each silicic acid has a unique physical and chemical structure with respect to distribution and quantity of the surface-active silanol groups, such structure being dependent on the history of the preparation[29].

Analysis of the methyl esters formed when lecithin is passed through the series of similar columns (Table I) indicates that the family of fatty acids involved in transesterification varies to some degree on each column. However, no obvious specificity towards a given fatty acid is present. In order to explain these findings, more systematic research on the subject is required. It is also difficult to explain the more pronounced formation of lysolecithin at a lower temperature.

Lysolecithin is a phospholipid with important *in vitro*, and (apparently) *in vivo*, properties. These are connected with its ability to interact with synthetic and biological membranes. Its presence has been frequently reported in living tissues, but there are considerable discrepancies among the published figures[18–27]. These investigators, with the exception of COLLINS *et al.*[19], have all made use of silicic acid chromatography for phospholipid fractionation. COLLINS used counter-current distribution, and failed to detect lysolecithin. Our results suggest that some of the discrepancies could be the effect of one or more of the variables that influence the transesterification of lecithins on silicic acid columns.

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SUMMARY

Natural and synthetic lecithins were found to undergo transesterification and hydrolysis during chromatography on silicic acid columns. Since these reactions are of importance in relation to the detection of lysolecithin in animal tissues, some of the factors influencing the reactions were studied.

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