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The analysis of tissue phospholipids: hydrolysis procedure and results with pig liver

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SUMMARY

A procedure for the hydrolysis of phospholipids is described. The effect of alkali concentration, organic solvents, and length of hydrolysis have been studied. The initial reaction in the preferred solvent system appears to be a methanolysis. The phospholipids are converted quantitatively into water-soluble phosphates, which are separated and estimated by ion-exchange chromatography. In this way phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol may be determined in liver tissue.

Previous investigations (1, 2) on the separation of mono- and diesters of phosphoric acid by means of ion-exchange resins have shown that in principle this method can be applied to the analysis of the total glycerophosphatides of animal tissues. The method is mainly the extension of the work of Dawson (3) and involves mild alkaline hydrolysis of total phospholipids, extraction of the resulting water-soluble esters, and separation of the latter on ion-exchange resins, using a sodium tetraborate-ammonium formate system for elution (4).

A study of Dawson's method (3) showed that only 60% to 65% of the glycerophosphatide phosphorus was recovered in the aqueous phase. The low recovery is no disadvantage when only the specific activity of radioactive lipids is to be determined, but may introduce errors when the relative proportions of the constituent phospholipids have to be determined. The loss of phosphorus, occurring during the preparation of glycerylphosphoryl diesters from total phospholipids, might not be the same from all diesters.

The present work describes studies on the mode of alkaline hydrolysis of total phospholipids, gives further details of the chromatographic separation of the diesters, and reports a procedure which gives quantitative recovery of glycerophosphatide phosphorus in the form of diesters.

MATERIALS AND METHODS

Analytical methods and details of ion-exchange chromatography were described previously (1, 2).

Pure sphingomyelin was prepared according to Klenk and Rennkamp (5). Analytical determinations were as follows: N, 3.1%; P, 3.6%; molar ratio of P:choline, 1.0:1.0 (theory 1:1); molar ratio of P:N, 1:1.90 (theory 1:2). Esterified fatty acids could not be detected.

Esterified fatty acids were determined by the method of Stern and Shapiro (6). Samples of 0.2 to 0.4 ml were taken, made up to 3 ml with alcohol-ether 3/1(v/v), and then treated as described by Stern and Shapiro.

Tissues were treated in the following manner for the extraction of total lipids. The fresh tissue was homogenized in a Waring blendor with at least 10 volumes of 5% trichloroacetic acid. The precipitate was collected by centrifugation and washed 6 times with a similar volume of ice-cold, 5% trichloroacetic acid, to remove water-soluble phosphates. The precipitate was finally washed with 10 volumes of distilled water to reduce the acidity and extracted for 1 hour at room temperature with 10 volumes of chloroform-methanol 1/1(v/v). The insoluble material was removed by filtration through a plug of glass wool and the filtrate taken to dryness *in vacuo*. The dry residue was extracted with chloroform for 2 hours and the insoluble protein filtered off.

Hydrolysis of such lipid mixtures was carried out at about 20° in graduated centrifuge tubes. The volume of the solvent mixture was 1 to 2 ml and methanolic NaOH was added. The reaction was stopped by adding 3 ml of distilled water, 3 ml of ether, and enough 1 N HCl to give a final pH of 1.

The mixture was shaken and centrifuged. Aliquots of the aqueous phase were taken for phosphate analysis.

Preparation of Crude Phospholipids. One pig liver was minced, partially dried with 5 volumes of acetone at 4°, and then extracted with chloroform-methanol 2/1(v/v). The extract was taken to a small volume under reduced pressure and phospholipids were precipitated by adding 10 volumes of acetone. The precipitate was dissolved in chloroform.

RESULTS

Choice of Solvent System. A comparison between the hydrolysis of pig liver phospholipids in chloroform-methanol 1/8(v/v), chloroform-methanol 4/5(v/v), and chloroform-methanol-ethanol-ether 1/1/3/2(v/v) showed that there was little difference. The water-soluble phosphates recovered in the aqueous phase after 10 minutes hydrolysis with 0.2 N alkali amounted to 84%, 89%, and 88%, respectively, of the phospholipid added. The second mixture of chloroform-methanol was taken in subsequent experiments, since it was then easier to obtain two phases when water and ether were added after hydrolysis.

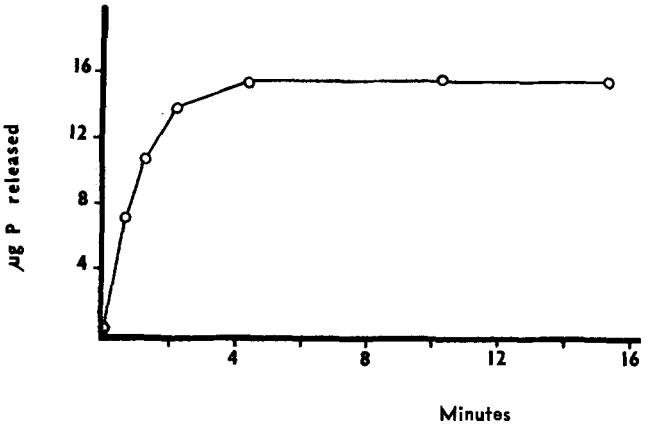

FIG. 1. Time study of phospholipid hydrolysis. Details in text. Curve records μg phosphorus in a 2 ml aliquot of the final aqueous phase.

Time Study. In these experiments, 0.2 ml of crude phospholipid in chloroform (174 μg P/ml), 0.4 ml of chloroform-methanol 2/1(v/v), and 0.4 ml of 0.5 N methanolic NaOH were kept at room temperature for different periods of time. At the end of hydrolysis, 0.3 ml of N HCl, 3 ml of water, and 5 ml of ether were added. The aqueous phase was analyzed for water-soluble phosphate. As can be seen in Figure 1, no further release of water-soluble phosphate occurred after 4 minutes of hydrolysis. If the phosphate values shown in Figure 1 are corrected for the total volume of the aqueous phase (4.2 ml), 89% of the phospholipid phosphorus became water-soluble during hydrolysis. When the same phospholipid preparation was analyzed for alkali-stable phospholipids according to the method of Schmidt *et al.* (7), 10.8% of the total phospholipid phosphorus was found to be stable (see also below). This suggests the presence of slightly more than 10% of sphingomyelin plus plasmalogens in the crude phospholipid preparation used.

Concentration of Alkali. In a similar experiment to that recorded in the previous section, the concentration of alkali was varied and hydrolysis was carried out for 10 minutes. The final concentrations of alkali were 0.2, 0.1, 0.05, and 0.025 N. No significant change in the amount of water-soluble phosphates was found.

Since it was observed that phospholipid mixtures have some buffering capacity, the amount of phospholipid taken was varied, keeping the alkalinity constant. The phospholipid phosphorus added was 10.7, 21.4, 49.5, and 84.4 μmoles per test (total volume of 1.0 ml containing 50 μmoles of NaOH). Between 89% and 94% of the phospholipid phosphorus was recovered in the first three experiments, the difference being within experimental error. In the experiment with the highest phospholipid concentration, only 35% of the phosphorus added was recovered in the aqueous phase. It seems, therefore, important to relate the relative amounts of alkali and phospholipid rather than to hydrolyze at a given final concentration of alkali. The above experiments suggest that complete hydrolysis of phospholipid occurred at molar ratios of phosphorus to alkali up to 1.

Esterified Fatty Acid Values. In some experiments the hydrolysis of phospholipids was followed by determination of the fatty acid ester linkages, using the hydroxamic acid method (6). Four ml of phospholipid in chloroform (174 μg P/ml), 4 ml of chloroform-methanol 2/1(v/v), and 2 ml of 0.5 N methanolic NaOH were mixed, and 0.4 ml samples were taken at intervals for ester determination. Figure 2, curve *A*, shows that no change in ester value occurred during the first hour of hydrolysis, though almost 90% of the phospholipid phosphorus had become water-soluble during the first 4 minutes of hydrolysis. When a small amount of water was added to the hydrolyzate (curve *B*, 4 ml phospholipid solution, 4 ml of chloroform-methanol, 1 ml of water, and 1 ml of N methanolic NaOH), the ester value had decreased to 50% of its original value during 1 hour.

In view of the apparent discrepancy between ester

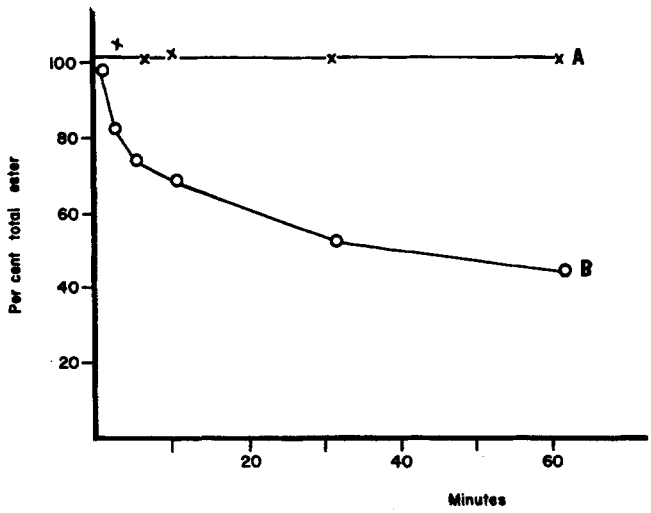

FIG. 2. Change in carboxylic ester linkages during hydrolysis. Curve *A*, usual conditions. Curve *B*, water added. Details in text.

values and water-soluble phosphates, a slightly larger amount of phospholipid was hydrolyzed for 10 minutes in chloroform-methanol, using a slight molar excess of alkali. At the end of hydrolysis, water and ether were added, and enough acid to bring the pH of the mixture to 1. The aqueous phase contained 92% of the phospholipid phosphorus added and ester linkages could not be detected; 82% of the ester linkages present at the beginning of the reaction was recovered in the organic phase. After careful washing of this phase with water, titration showed that on a molar basis, 12% of the fatty acids originally present had been recovered as free fatty acid. This last result is not in agreement with the fact that the ester values did not change during 1 hour's hydrolysis. It should be remembered, however, that at the end of hydrolysis, water and acid were added, which might explain the difference.

The results suggested that the long-chain fatty acids of phospholipids are "trapped" as esters, the mode of breakdown being a methanolysis. The possibility that glycerides were formed during alkaline hydrolysis was excluded by determination of glycerol. In this experiment the ether phase obtained after mild alkaline hydrolysis, and containing 440 μmoles of ester linkages, was hydrolyzed in 1.5 ml of 0.5 N ethanolic KOH for 16 hours at 37°. No ester linkages were detected after this second hydrolysis. Free glycerol was determined on the hydrolyzate (8). Assuming that diglyceride was the only source of ester linkages in the ether phase obtained after the first mild alkaline hydrolysis, 220 μmoles of glycerol should have been present. Actually, 0.19 μmole, or 0.1%, of the expected was found. In the presence of water (Fig. 2, curve *B*), the primary reaction again seems to be methanolysis followed by a slow hydrolysis of the methyl esters. To obtain more evidence about the methyl esters, an aliquot of the organic phase was treated with a small amount of alumina to remove the free fatty acids present and applied to a gas-liquid chromatography column. The results are shown in Figure 3. Since glycerides were not found, it appears that even after addition of water and acid (see above), about 88% of fatty acids can be recovered as methyl esters.

Experiments with Hydroxylamine. The conditions of Stern and Shapiro (6) for the determination of esterified fatty acids (3 ml of organic solvent containing phospholipid, 0.5 ml of 2 M aqueous hydroxylamine hydrochloride, and 0.5 ml of 3.5 M aqueous NaOH), but without the addition of HCl and ferric chloride, also gave good recovery of phospholipid phosphorus in the aqueous phase. Experiments were also made with free hydroxylamine. This was prepared by the addition of the calculated quantity of the hydroxylamine hydrochloride to sodium methoxide in dry methanol. The precipitated sodium chloride was filtered off. Using this hydroxylamine without addition of alkali, only 4% to 6% of phospholipid phosphorus became water-soluble. Hydroxamic acids could not be detected. If the

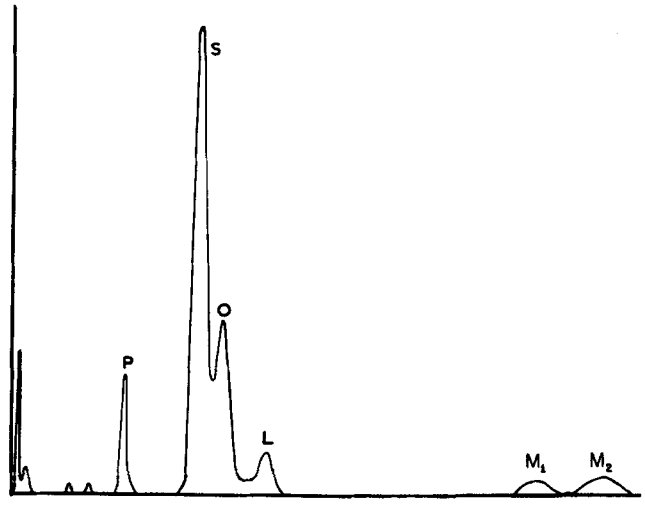

FIG. 3. Gas-liquid chromatography of ether-soluble hydrolysis products. The Martin apparatus was used (Abbotsbury Laboratories), with a gas-density meter (Martin and James, 9). The column was 1.5 m long and had a 3.5 mm bore. Nitrogen was the carrier gas and the stationary phase consisted of polyethylene glycol adipate on Celite®. The running temperature was 197°. Further details are given by Sammons and Wiggs (10). *P* = palmitic acid; *S* = stearic acid; *O* = oleic acid; *L* = linoleic acid; M_1, M_2 = C_{20} acids.

solution of free hydroxylamine contained traces of sodium methoxide, or if dilute sodium methoxide alone was used, good recoveries of phospholipid phosphorus resulted.

The Behavior of Sphingomyelin. In the experiments reported so far, the water-soluble phosphates always corresponded to significantly less than 100% of the phospholipid added. Sphingomyelins are known to be resistant to alkaline hydrolysis (7). A sample of highly purified sphingomyelin was treated in the way described above for phospholipids, except that chloroform was used instead of ether in the final extraction. Using 7.2 mg sphingomyelin, 6 ml 0.5 N NaOH in methanol, 12 ml chloroform, and 6 ml chloroform-methanol 2:1, the final aqueous phase (54 ml), after one extraction with chloroform, was analyzed for phosphate. None was detected in aliquots of 4 ml. When ether was used to extract the hydrolyzate, small amounts of phosphate were detected in the aqueous phase. Most of the sphingomyelin appears to collect at the ether-water interface, since it is insoluble in both solvents, but a little presumably dissolves in the water, accounting for the observed phosphate.

The crude pig liver phospholipid which usually gave about 90% recovery of its phosphate in water-soluble form when hydrolyzed by the above method, was also analyzed for sphingomyelin by the method of Schmidt *et al.* (7). A typical estimation gave 10.8% of total lipid P in alkali-stable combination. This figure is high for liver sphingomyelin, but the preparative method may have caused some enrichment of this lipid relative to the other phospholipids.

Treatment with Cation-Exchange Resins After Hydrolysis. In Dawson's original procedure (3), the sodium hydroxide was removed from the phospholipid hydrolyzate by Amberlite® IRC 50. A similar carboxylic resin, Zeo-Karb 226, was used in our earlier studies based on Dawson's method (1, 2). There was a considerable loss of phosphate at this stage unless the resin was washed with a large volume of water. Since the hydrolyzate was loaded onto the Dowex 1 column in the presence of sodium borate, there was no need to remove the small amount of sodium hydroxide involved in the modified hydrolysis above. Instead, boric acid was added to give the required 0.005 M sodium borate concentration. This avoided the loss of phosphate esters.

Effect of Lipid-Extracting Solvents. Carbon tetrachloride, ether, petroleum ether, and isobutanol were used successively in Dawson's method. There was little loss of phosphate esters from the aqueous phase during these extractions, but even after use of all four solvents, some colored material always appeared at the top of the column when the final aqueous phase was chromatographed.

Omitting the treatment with carboxylic resin leaves the diluted hydrolyzate alkaline, so extraction of fatty acids into an organic solvent cannot be expected. The extraction procedure was therefore simplified. Two extractions with ether were performed and the interfacial material sometimes noticed after centrifuging was rejected with the ether. The Dowex columns showed less discoloration than in the original procedure, and the sodium salts of any fatty acids present were not retained by the resin.

Comparison of the Original and Modified Procedure by Resin Chromatography. Samples of the same pig liver phospholipid mixture were hydrolyzed by either the original or the modified method and loaded onto Dowex 1 columns for the separation of the resulting phosphodiesters. Details of two such experiments are given below.

(*a*) *Original Procedure.* A volume of 5 ml of phospholipid solution in chloroform (total P, 17 mg) was mixed with 63 ml methanol, 3 ml chloroform, 9 ml water, and 20 ml N NaOH in methanol. The mixture was incubated at 37° for 30 minutes. At the end of this period 100 ml ice water was added, and sufficient Zeo-Karb 226 (16 to 50 mesh) to bring the pH to 7. The resin was then filtered off through a glass-wool plug and washed with 100 ml deionized water. The combined hydrolyzate and washings were next extracted with 200 ml volumes of carbon tetrachloride, ether, light petroleum (40° to 60°), and isobutanol. The final aqueous layer was made to 250 ml, sodium tetraborate solution being added to give a concentration of 5 mM. The solution contained 11.2 mg phosphorus (66% recovery) and was applied directly to a Dowex 1×2 column (100 to 200 mesh, formate form, 1×20 cm). The eluate was analyzed for P and the following eluting mixtures were applied to the column: 5 mM sodium tetraborate-10 mM ammonium formate (1.5 liter), 5 mM sodium tetraborate-40 mM ammonium formate (500 ml), 5 mM sodium tetraborate-80 mM ammonium formate (1 liter), and 5 mM sodium tetraborate-0.15 M ammonium formate (1 liter). The elution pattern is shown in Figure 4.

(*b*) *Modified Procedure.* Four ml phospholipid solution (total P 13.6 mg), 3 ml chloroform-methanol 2/1(v/v), and 2 ml 0.5 N NaOH in methanol were mixed and allowed to stand for 15 minutes at room temperature. Sixty ml deionized water and 40 ml ether were then added and, after shaking well, the mixture was centrifuged. The clear ether layer and any inter-

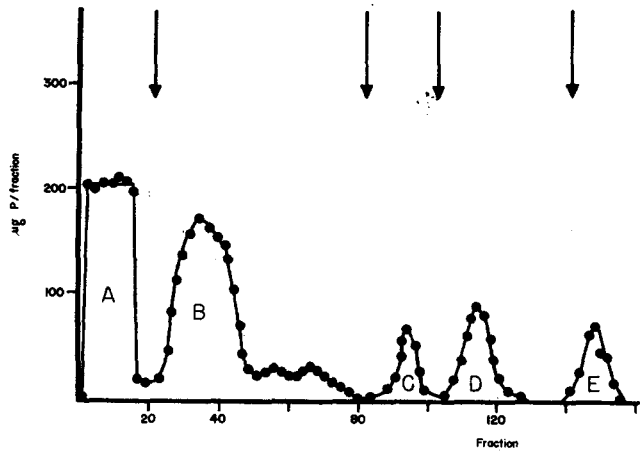

FIG. 4. Dowex 1 separation of pig liver phospholipid hydrolysis products. Details in text. Eluting mixtures: Start to first arrow, loading of column; first to second arrow, 5 mM sodium tetraborate-10 mM ammonium formate; second to third arrow, 5 mM borate-40 mM formate; third to fourth arrow, 5 mM borate-80 mM formate; fourth to fifth arrow, 5 mM borate-150 mM formate. Peaks: *A*, glycerylphosphorylcholine; *B*, glycerylphosphorylethanolamine; *C*, glycerylphosphorylinositol; *D*, glycerylphosphorylserine; *E*, unknown.

facial material were removed by aspiration and the lower layer made to 500 ml with water, after the addition of 20 ml 0.1 M sodium tetraborate and 5 ml 0.1 M boric acid. The solution contained 12.2 mg total P (90% recovery, i.e., loss of alkali-stable phospholipid phosphorus, see above) and was applied to a Dowex 1 column and treated exactly as described in section (*a*) above. The elution pattern is given in Figure 5.

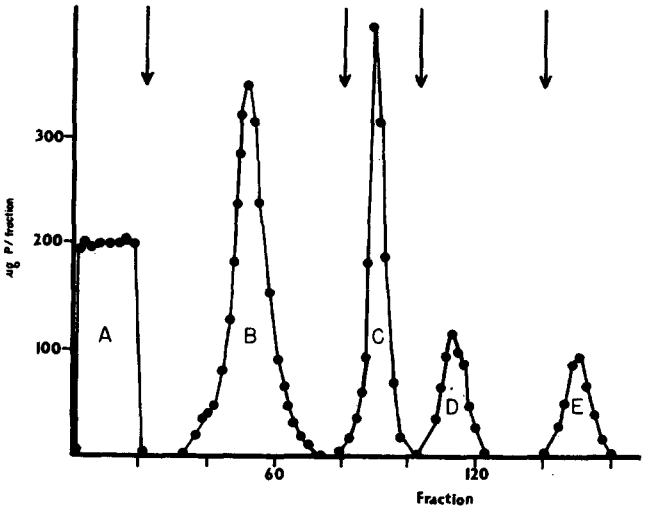

FIG. 5. Dowex 1 separation of pig liver phospholipid hydrolysis products (modified method). Details in text. For eluting mixtures and identity of peaks see Figure 4.

Figures 4 and 5 show that the peaks are better separated when the new method is used, and the small peaks following peak *B* do not appear. The reason for this is unknown. Peak *A* represents the last of the glycerylphosphorylcholine which passes right through the column. Peaks *B*, *C*, and *D* represent glycerylphosphorylethanolamine, glycerylphosphorylinositol, and glycerylphosphorylserine, respectively. Peak *E* is unidentified.

Application of the Method to Fresh Tissue. An amount of tissue containing 10 to 15 mg phospholipid phosphorus was homogenized for 1 minute in a Waring blendor with 10 volumes of 5% trichloroacetic acid. The homogenate was centrifuged and the precipitate resuspended in a similar volume of the same acid. The washing was repeated three times and followed by one washing with distilled water. The tissue was then extracted at room temperature for 1 hour with 10 ml of 1:1 chloroform-methanol per g fresh tissue, and the coarser solids removed by filtration through a glass-wool plug. The filtrate was dried *in vacuo* and the residue of lipid and proteolipid protein extracted with dry chloroform. In this way protein was removed, and the extract contained all the tissue lipids. When necessary, it was concentrated *in vacuo* to a volume of 10 ml. Three ml of methanol and 2 ml of 0.5 N methanolic NaOH were then added. After 15 minutes at room temperature, 50 ml of water and 50 ml of ether were added. The mixture was shaken and centrifuged. The ether layer and any interfacial material were removed. To the lower layer 5 ml of 0.1 M boric acid and 20 ml of 0.1 M sodium tetraborate were added, and the volume was made to 500 ml with distilled water.

This solution was analyzed for phosphorus and loaded onto a Dowex 1 column, as described above. The phosphorus content of the eluate must be determined, since glycerylphosphorylcholine passes right through the column.

The elution mixture cited above may be used, with modification, where a tissue contains large amounts of a specific phospholipid. Preliminary results indicate that the phosphatides of a wide range of tissues may be analyzed in this way (e.g., liver, brain, and intestine of the rat). Several unidentified peaks have been observed.

DISCUSSION

The method most commonly used at present for the separation of phospholipid hydrolysis products is two-dimensional paper chromatography. The resin column

method offers several advantages over this procedure, though relatively large quantities of phospholipid are required (5 to 10 mg of phosphorus). The advantages are that it can be used after a hydrolysis which is simpler and quicker to perform and which gives quantitative recovery of alkali-labile phospholipid phosphorus. There is a possibility that the use of trichloroacetic acid may cause breakdown of plasmalogens to give lysophospholipids, which are alkali-labile. In the case of liver, where little plasmalogen is found, no difficulty arises. But with tissues such as brain, a milder extraction procedure may be required. Preliminary experiments have been made using chloroform-methanol 2/1(v/v) to extract fresh liver tissue, but the recovery of certain phospholipids is not the same using this method as with the trichloroacetic acid method. The stability of plasmalogens under the various conditions used is being investigated.

The resin method can also be used for the isolation and chemical identification of hydrolysis products (1, 2). By scaling up the amounts, minor phospholipid constituents of tissues can be studied in detail. In addition, it gives the exact composition of a tissue phospholipid mixture, provided that all the peaks obtained represent pure compounds. For the tissues studied so far, this seems to be so. Finally, the hydrolysis products of phosphatidylinositol and "diphosphoinositide"[1] are readily separated. In paper chromatography both run slowly and tend to overlap. The use of dilute alkali and short hydrolysis ensures that the diesters produced from phospholipids do not hydrolyze further to monoesters (2).

The initial products of alkaline degradation of phosphatides in anhydrous chloroform-methanol solution appear to be methyl esters of fatty acids. This is shown by the gas-liquid chromatography of extracts from the reaction mixture and also by the failure of the ester values to decrease under these conditions (Fig. 2). Thus the initial reaction seems to be methanolysis.

Table 1 shows the phosphate distribution among the various peaks using the original and modified methods on a phospholipid mixture prepared from pig liver. This mixture does not represent the total phospholipids of the tissue. Two further small peaks are seen when the total lipids of rat liver are hydrolyzed by the modified method. One of these may arise from "polyglycerophospholipid." In addition, figures from the literature for rat liver phospholipids are given for comparison.

The recovery of phosphate in the peaks is higher by the new method, partly because of tailing and losses on the column when the original method is used. The other reason for better recovery in peaks *A* to *E* is the absence of the small peaks following glycerylphosphorylethanolamine (Fig. 4). These peaks may arise from unknown minor components; however, if they are artifacts, the new method has a further advantage.

A study of this method of the phospholipid composition of rat liver and intestine is in progress.

[1] P. Kemp and J. N. Hawthorne, unpublished observations.

TABLE 1. ANALYSIS OF PHOSPHOLIPIDS FROM LIVER *

	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Phosphatidylinositol	Unknown peak	Total
Original hydrolysis, pig liver †	40	21	6.1	3.5	2.9	73
Modified hydrolysis, pig liver	43	25	6.6	13.7	3.7	92
Rat liver (11) ‡	43	27.6	6.6	—	—	77
Rat liver (12) ‡	39–65	27.5	11.3	—	—	—

* Per cent of total lipid phosphorus present in each component.
† Crude preparation of phospholipids (see Methods).
‡ Number in parenthesis is reference.

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