Isolation of Native Plasmalogens *, **

O. RENKONEN

Department of Serology and Bacteriology, University of Helsinki, Finland

A general method has been developed for the isolation of native plasmalogens. The method is based on selective deacylation of natural mixtures of the plasmalogens and the corresponding diacyl phosphatides by mild alkaline treatment, which destroys the plasmalogens more slowly than the diacyl phosphatides. Native ethanolamine plasmalogens were thus isolated for the first time in fairly pure form. Native choline plasmalogens could also be isolated by the method described. The plasmalogens isolated still remained contaminated with small amounts of the corresponding acylated alkoxy phosphatides, which were deacylated by alkali at about the same rate as the plasmalogens.

Recently it has become evident that most natural glycerophosphatides are mixtures of two or three different types of lipids differing only in the oxygen grouping of one of their fatty chains. Thus the "lecithins" of human serum, for example, are a mixture of phosphatidylcholines, choline plasmalogens and acylated α-alkoxy-glycerophosphorylcholines [1].

The studies of Debuch [2], Hanahan and Watts [3], Gottfried and Rapport [4], as well as those in our laboratory [1] have clearly established the value of selective hydrolysis in the isolation of the pure types of the native phosphatides from such mixtures. These studies have already led to general methods for the isolation of different diacyl phosphatides [2] and native alkoxy phosphatides [1,3], but for native plasmalogens no general methods have yet become available although a specific one for choline plasmalogens has been described [4]. — Gottfried and Rapport found recently that choline plasmalogens of ox-heart are deacylated more slowly than the corresponding phosphatidylcholines by rattlesnake venom. This finding enabled them to isolate a part of the choline plasmalogens, which was the first fairly pure plasmalogen preparation to be described [4]. However, this enzymic method, although a very interesting one, cannot be adapted to the preparation of all different plasmalogens, since only few types of phosphatides are deacylated by snake venoms.

Recent work in our laboratory suggested, contrary to some observations of Hanahan and Watts[3], that alkali may also deacylate the plasmalogens more slowly than the corresponding diacyl phosphatides[1]. The validity of this observation, which seemed to provide a more general way of preparing native plasmalogens than the enzymic method was tested by subjecting some well-known mixtures containing plasmalogens to partial alkaline deacylation.

This report describes the isolation of fairly pure choline and ethanolamine plasmalogens from partial alkaline hydrolysates of "lecithin" of ox-heart and "ethanolamine cephalin" of ox-brain.

EXPERIMENTAL

Analytical methods. Determination of phosphorus[5], carboxylic esters[6], and glycerol[7] was carried out by the recorded procedures. The estimation of diacyl phosphatides, plasmalogens and alkoxy phosphatides was carried out by a modification of the "phospholipid partition" method of Schmidt *et al.*[8], the "stable" (lipid soluble) and "labile" (water soluble) phosphorus after mild acid, mild alkaline, as well as mild acid and alkaline hydrolysis being estimated by methods developed in our laboratory[9]. However, the mild alkaline hydrolysis was carried out with 0.05 N NaOH.

Comments on the "phospholipid partition" method for the analysis of the different types of phosphatides. The analysis of diacyl phosphatides, plasmalogens and alkoxy phosphatides in preparations free from sphingomyelins by the method of "phospholipid partition "[8,10] is based on the combined use of two methods of hydrolysis. Mild alkaline treatment converts the diacyl phosphatides to water soluble phosphates, whereas the phosphorus of plasmalogens and alkoxy phosphatides is believed to remain lipid soluble. Combined mild hydrolysis with acid and alkali, on the other hand, is believed to convert the phosphorus of both the diacyl phosphatides and of the plasmalogens to water soluble form, whereas that of the alkoxy phosphatides is considered lipid soluble.

Although there is good evidence for the correctness of these assumptions as regards the diacyl phosphatides and the alkoxy phosphatides[1,8-12], the exact behaviour of the plasmalogens is still uncertain. Indeed, the "phospholipid partition" method has been criticized by Pietruszko and Gray[13], who believe that a part of the plasmalogen phosphorus may also become water soluble during the mild alkaline hydrolysis.

The availability of pure or fairly pure samples of phosphatidylcholines, choline plasmalogens, and acylated α-alkoxy-glycerophosphorylcholines has now allowed a direct comparison of the behaviour of the three basic types of glycerophosphatides during the hydrolytic procedures of the "phospholipid partition" method.

Table 1. Hydrolysis of "twochain" derivatives of glycerophosphorylcholine.

Lipid [a]	Mild alkaline hydrolysis[b]		Mild acid and alkaline hydrolysis[b]	
	Stable P	Labile P	Stable P	Labile P
	%	%	%	%
Phosphatidylcholine	1	99	1	99
Choline plasmalogen	95	5	16	84
Acylated alkoxy-glycero-phosphorylcholine	95	5	96	4

[a] The origin of the lipid samples is described in the text.
[b] Uncorrected result of hydrolysis.

Table 1 shows that the three pure lipid types can be differentiated from each other by the "phospholipid partition" method in the way expected. However, the figures of Table 1, as well as the analogous findings on "monochain" derivatives of glycerophosphorylcholine reported earlier [9] show that our methods of hydrolysis tend to give too low values for the "stable" fractions. This was found to be caused mainly by incomplete fractionation of glycerophosphorylcholine and its "monochain" derivatives after the hydrolysis. Our method for the fractionation of the hydrolysates is based on the use of the salt containing partition system of Folch *et al.*[14] Direct measurement of the partition coefficient of lysophosphatidylcholine in this system showed that only about 93 % of its phosphorus remained in the lipophilic fraction. The other "monochain" derivatives of glycerophosphorylcholine probably have very similar partition coefficients. Thus it is obvious that the results of the hydrolyses recorded in Table 1 must be corrected by adding to the "stable" fractions 7 % of their respective phosphorus contents, and by subtracting the same amount of phosphorus from the corresponding "labile" fractions. After this correction satisfactory figures are obtained for the three pure lipids of Table 1.

Also mixtures of these three lipid types can be analyzed fairly accurately by the "phospholipid partition" method. This is exemplified by the analysis of the sample of ox-heart "lecithin" used in this study. Its plasmalogen content estimated by the hydrolytic method was 33 %, which was in fair agreement with the result (32 %) obtained by the iodine method of Gottfried and Rapport [4].

The partition coefficient of lysophosphatidylethanolamine was very nearly the same as that of lysophosphatidylcholine. Accordingly the above corrections must be applied to the results of hydrolysis also in the analysis of "ethanolamine cephalins".

Chromatographic methods. Column chromatography on silicic acid [15], alkali free aluminum oxide [16], and DEAE cellulose [17] was carried out at room temperature with stepwise gradient elution with chloroform-methanol mixtures. Chromatography on papers impregnated with silicic acid was carried out as described by Marinetti *et al.*[18], staining with Rhodamine 6 G. Thin layer chromatography (TLC) on Kieselgel G (E. Merck AG) was performed as described by Wagner *et al.*[19], staining by charring with 50 % sulphuric acid.

Phospholipid preparations. "Lecithin" of ox-heart was isolated from a total lipid extract of fresh tissue by repeated chromatography on silicic acid and alkali free alumina. The final preparation was homogeneous on TLC and on paper chromatography, and showed the same mobility as phosphatidylcholines. It was free of sphingomyelins. Chemical analysis showed that it contained 63 % phosphatidylcholines, 33 % choline plasmalogens, and 4 % acylated alkoxy phosphatides.

"Ethanolamine cephalin" of ox-brain was prepared by subjecting a "Folch V" fraction [20] to chromatography on DEAE cellulose. The final preparation was homogeneous on TLC and on paper chromatography, and showed the same mobility as pure phosphatidylethanolamines. Chemical analysis showed that it contained 40 % phosphatidylethanolamines, 56 % ethanolamine plasmalogens, and 4 % acylated alkoxy cephalins.

Pure phosphatidylcholines were isolated by repeated chromatography of egg yolk phosphatides on silicic acid and alumina columns.

The sample of acylated α-alkoxy-glycerophosphorylcholines was isolated from normal human serum [1].

The lysophosphatidylcholine preparation used in the measurement of the partition coefficient was isolated from human serum essentially as described elsewhere [21]. The sample of lysophosphatidylethanolamine used in similar experiments was kindly supplied by T. H. Bevan.

The samples of phosphatidylethanolamine used as chromatographic markers were a dioleoyl derivative synthetized by E. Baer and kindly supplied by G. V. Marinetti, and a dipalmitoyl derivative obtained from Fluka AG, Buchs, Switzerland.

RESULTS

Isolation of native choline plasmalogens. To a solution of "lecithin" of ox-heart (7.0 mg P) in 300 ml of chloroform-methanol (1:1) was added 50 ml of 0.35 N NaOH in 96 % methanol, and the mixture was incubated for 45 min at 20°. Then 148 ml of water, 2 ml of methanol, and 250 ml of chloroform were
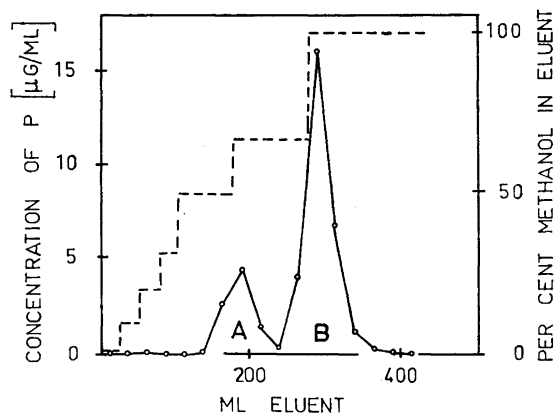
Fig. 1. Silicic acid chromatography of the stable phosphatides in the partial alkaline hydrolysate of ox-heart "lecithin". Lipid added to column: 0.956 mg phosphorus; recovered: 0.932 mg phosphorus. Dashed line: Composition of eluent.

added, and the liquid layers of the resulting solvent mixture the partition system of Folch *et al.* were shaken well and centrifuged. The chloroform layer was separated, washed with fresh aqueous phase and dried over Na_2SO_4. The alkali stable lipids (2.4 mg P) thus obtained were then fractionated by chromatography.

Silicic acid chromatography of a sample (0.96 mg P) of this preparation gave the elution curve shown in Fig. 1. The fractions comprising Peak A contained native choline plasmalogens, whereas those of Peak B contained a mixture of various "monochain" derivatives of glycerophosphorylcholine, the principal component being choline lysoplasmalogens.

The rest of the alkali stable lipids (1.43 mg P) were chromatographed on a column of aluminium oxide, which gave essentially similar results to the silicic acid column.

The fractions comprising the plasmalogen peaks of the two chromatograms were pooled and subjected to characterization without any further purification.

The total yield of native plasmalogens isolated amounted to 18 μmoles, which is 24 % of the plasmalogen content of the hydrolyzed sample.

Characterization of the native choline plasmalogens. The preparation obtained was homogeneous on TLC and showed the same mobility as phosphatidylcholines, but differed clearly from sphingomyelins. Upon analysis it showed 0.982 equivalents of carboxylic ester and 0.908 moles glycerol per 1.00 g. atom of phosphorus. It did not contain any phosphatidylcholine as 95 % of its phosphorus * remained "stable" after renewed alkaline hydrolysis.

Mild acid hydrolysis ** of the preparation released only 7.5 % of water soluble phosphorus. Thus it probably did not contain appreciable amounts of

---

* Uncorrected result of hydrolysis.
** The hydrolysis was carried out as described previously [9].

"lecithins" with two vinyl ether chains [22]. The stable lipids of the acid hydro-
lysate were subjected to chromatography on silicic acid, which gave again a
"lecithin-like" fraction and a "lysolecithin-like" one. The latter contained
79 % of the total phosphorus in the original plamalogen preparation. This
fraction consisted of true lysophosphatidylcholines formed from the plasma-
logens by the acid treatment. Their identity was established by TLC, by their
equimolar content of carboxylic ester and phosphorus, and by their "stability"
to mild acid hydrolysis, and "lability" on mild alkaline hydrolysis.

The "lecithin-like" fraction obtained from the acid hydrolysate of the plas-
malogen preparation was tentatively identified as acylated alkoxy-glycero-
phosphorylcholine contaminated by small amounts of sphingomyelins. — The
preparation was nearly homogeneous on TLC, the principal component show-
ing the same mobility as phosphatidylcholine, but it released only traces of
free glycerol on strong acid hydrolysis.

Analytical determination of acid and alkali stable phosphatides showed
that the plasmalogen preparation contained about 17 % alkoxy phosphatides
and sphingomyelins.

All these findings show that the plasmalogen preparation isolated from
the partial alkaline hydrolysate of "lecithin" of ox-heart was a mixture con-
taining about 83 % native choline plasmalogens and about 10—15 % acylated
alkoxy-glycerophosphorylcholines and traces of sphingomyelins. — The pre-
sence of alkoxyphosphatides in ox-heart "lecithin" was recently observed also
by Pietruszko and Gray [13].

Isolation of native ethanolamine plasmalogens. The pure "ethanolamine
cephalin" of ox-brain (0.54 mg P) was deacylated exactly like the "lecithin"
sample but the solvent volumes were reduced 25-fold, and the duration of the
alkaline incubation was reduced to 23 min, because of the larger relative
amount of plasmalogens in this sample. The alkali stable lipids obtained (0.30
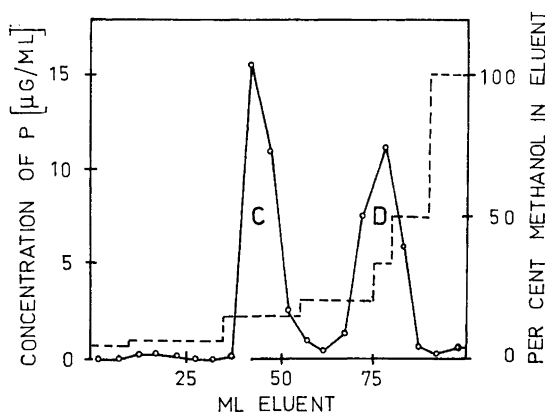mg P) were again fractionated on a column of silicic acid, which gave the

Fig. 2. Silicic acid chromatography of the stable phosphatides in the partial alkaline
hydrolysate of a purified "Folch V" fraction. Lipid added to column: 0.298 mg phos-
phorus; recovered: 0.294 mg phosphorus. Dashed line: Composition of eluent.

elution curve presented in Fig. 2. The fractions of Peak C contained native ethanolamine plasmalogens, whereas those of Peak D were again a mixture of "monochain" derivatives of glycerophosphorylethanolamine, the main component being ethanolamine lysoplasmalogen.

As Peak C contained 4.8 μmoles of phosphorus the native ethanolamine plasmalogens were isolated in a 49 % yield.

Characterization of the native ethanolamine plasmalogens. The plasmalogen containing fractions of Peak C were pooled and subjected to characterization without any further purification. The preparation obtained was homogeneous on TLC and showed the same mobility as authentic phosphatidylethanolamines. It showed upon analysis 1.01 equivalents of carboxylic esters and 1.02 moles of glycerol per 1.00 g. atom of phosphorus. Practically all of its phosphorus remained "stable" on mild alkaline hydrolysis (88 %) * and on mild acid hydrolysis (94 %) *. However, nearly all of its phosphorus (94 %)* became water soluble on combined mild hydrolysis with acid and alkali.

These findings show that the preparation of the native ethanolamineplasmalogens contained about 88 % plasmalogens, about 6 % acylated alkoxy cephalins and about 6 % phosphatidylethanolamines. It was improbable that it contained appreciable amounts of "cephalins" with two vinyl ether chains [22].

DISCUSSION

The findings presented above show that the diacyl derivatives of glycerophosphorylcholine and of glycerophosphorylethanolamine can be deacylated more rapidly than the corresponding plasmalogens by mild alkaline treatment. Although no exact kinetic measurements have yet been made, it seems that under the experimental conditions used in 20—25 min about 50 % of the plasmalogens are deacylated compared with more than 90 % deacylation of the diacyl phosphatides. This difference is large enough to allow the isolation of the native choline and ethanolamine plasmalogens from most of their natural mixtures with the corresponding diacyl phosphatides.

We believe that the method of alkaline deacylation can further be extended to the detection and preparation of other types of plasmalogens. The wide potential application range of alkaline deacylation and its easy use in the partial reactions seem to offer even greater promise than the use of phospholipase A.

However, the plasmalogens and the native alkoxy phosphatides seem to be deacylated at about similar rates under the experimental conditions used. Therefore the isolated plasmalogens remain contaminated by the alkoxy phosphatides if these are present in the original mixture. This is a real drawback in the isolation procedure suggested, as the alkoxy phosphatides seem to be more widely distributed in nature than previously believed.

Although the elimination of the plasmalogens from the alkoxy phosphatides [1] is as easy as from the diacyl phosphatides [2], the reverse elimination, the removal of the alkoxy phosphatides from the plasmalogens, and also from the diacyl phosphatides, is more difficult to achieve. Gottfried and Rapport

---

* Uncorrected result of hydrolysis.

thought that their sample of choline plasmalogens, prepared from ox-heart "lecithin" by the enzymic method, was uncontaminated [4]. However, at that time the presence of alkoxy phosphatides in ox-heart "lecithin" was not yet recognized, and therefore they did not test their preparation for the presence of the alkoxy phosphatides. In our opinion the analytical figures of Gottfried and Rapport suggest that their choline plasmalogens were also contaminated by acylated alkoxy-glycerophosphorylcholines.

No gas chromatographic analyses have yet been made to establish whether the alkaline deacylation of choline and ethanolamine plasmalogens proceeds at the same rate irrespective of the fatty chains of the molecules. Nevertheless we think that it is quite likely, because the plasmalogens of human serum seem to be deacylated at the same rate as those of ox tissues.

REFERENCES

1. Renkonen, O. *Acta Chem. Scand.* **16** (1962) 1288.
2. Debuch, H. *Z. physiol. Chem.* **304** (1956) 109.
3. Hanahan, D. J. and Watts, R. *J. Biol. Chem.* **236** (1961) PC 59.
4. Gottfried, E. L. and Rapport, M. M. *J. Biol. Chem.* **237** (1962) 329.
5. Bartlett, G. R. *J. Biol. Chem.* **234** (1959) 466.
6. Renkonen, O. *Biochim. Biophys. Acta* **54** (1961) 361.
7. Renkonen, O. *Biochim. Biophys. Acta* **56** (1962) 367.
8. Schmidt, G., Ottenstein, B., Spencer, W. A., Keck, K., Blietz, R., Papas, J., Porter, D., Levin, M. L. and Thannhauser, S. J. *A.M.A. J. Diseases Children* **97** (1959) 690.
9. Renkonen, O. *Biochim. Biophys. Acta* **59** (1962) 497.
10. Dawson, R. M. C. *Biochem. J.* **75** (1960) 45.
11. Carter, H. E., Smith, D. and Jones, D. N. *J. Biol. Chem.* **232** (1958) 681.
12. Hübscher, G., Hawthorne, J. N. and Kemp, P. *J. Lipid Res.* **1** (1960) 433.
13. Pietruszko, R. and Gray, G. M. *Biochim. Biophys. Acta* **56** (1962) 232.
14. Folch, J., Lees, M. and Sloane Stanley, G. H. *J. Biol. Chem.* **226** (1957) 497.
15. Lea, C. H., Rhodes, D. N. and Stoll, R. D. *Biochem. J.* **60** (1955) 353.
16. Renkonen, O. *J. Lipid Res.* **3** (1962) 181.
17. Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D. and O'Brien, J. S. *J. Am. Oil Chem. Soc.* **38** (1961) 544.
18. Marinetti, G. V., Erbland, J. and Kochen, J. *Federation Proc.* **16** (1957) 837.
19. Wagner, H., Hörhammer, L. and Wolff, P. *Biochem. Z.* **334** (1961) 175.
20. Folch, J. *J. Biol. Chem.* **146** (1942) 35.
21. Salminen, A., Renkonen, O. V. and Renkonen, O. *Ann. Med. Exptl. Biol. Fenniæ (Helsinki)* **38** (1960) 447.
22. Marinetti, G. V. *J. Lipid Res.* **3** (1962) 1.