THE PRODUCTS OF MILD ALKALINE AND MILD ACID HYDROLYSIS OF PLASMALOGENS

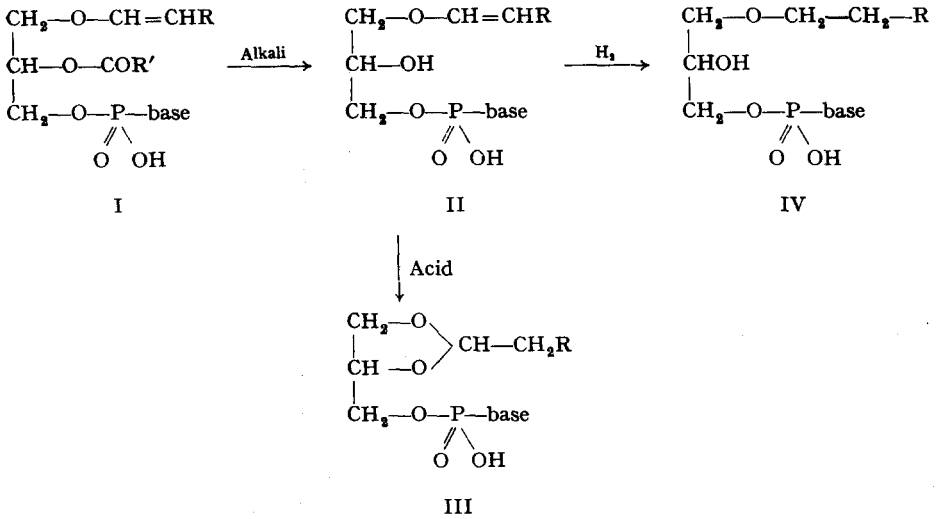
R. PIETRUSZKO AND G. M. GRAY
Lister Institute of Preventive Medicine, London (Great Britain)
(Received June 8th, 1961)

SUMMARY

Lysoplasmalogen with α,β-unsaturated ether structure is the sole product of the hydrolysis of choline and ethanolamine plasmalogens in 0.5 *N* aqueous and 0.1 *N* methanolic NaOH and also of the hydrolysis of choline plasmalogen with snake venom. Treatment of lysoplasmalogen with 90% acetic acid results in formation of cyclic acetal phospholipid. This compound has been isolated and characterized. Evidence for the occurrence of glyceryl ethers in lecithin and kephalin fractions from ox heart has also been obtained.

INTRODUCTION

Preliminary work in this laboratory[1] indicated that on hydrolysis of choline plasmalogen by alkali there was a loss of total aldehyde, as measured by a standard colorimetric technique of hydrolysis in 90% acetic acid at 50° and condensation with Feulgen reagent (method A); this was apparently due to the formation of a derivative more stable to acid than the expected lysoplasmalogen with the α,β-unsaturated ether structure, for the colorimetric aldehyde value increased if the hydrolysis at 50° was carried out in 90% acetic acid containing 0.5 *N* HCl and 0.0015 *N* $HgCl_2$ (method B). On fractionation of the product of alkaline hydrolysis on a silicic acid column the aldehydogenic material recovered had a molar ratio of aldehyde:P of 0.75:1 by method A, and 0.94:1 by method B, and an α,β-unsaturated ether:P ratio of 0.71:1 by the iodine method. It was concluded that on saponification of the native plasmalogen (I) with alkali two products were directly formed, a lysoplasmalogen (II) more labile to acid than the original plasmalogen and a cyclic form (III) more stable to acid than either the original plasmalogen or the standard dimethyl acetal of palmitaldehyde. It was considered possible that some cyclisation might occur as a result of some rearrangement of the α,β-unsaturated ether bonds during the actual process of deacylation of the adjacent hydroxyl group. This consideration was based to some extent on the fact that during alkaline hydrolysis there was a small real loss of total aldehyde, which indicated that the aldehydogenic linkage was not completely stable and some bond fission could occur. The cyclic form was isolated from the material by decomposition of the lysoplasmalogen with acetic acid and further fractionation on alumina.



Base = choline or ethanolamine.

DAVENPORT AND DAWSON[2], however, isolated the cyclic acetal of glycerylphosphorylethanolamine from kephalin plasmalogen by mild alkaline hydrolysis followed by acid hydrolysis and concluded that the cyclic compound was a secondary product formed by the action of acid on the lysoplasmalogen. We have verified that this is the correct interpretation by hydrogenation of the product of alkaline hydrolysis; this would lead to the formation of the glyceryl ether derivative (IV) with total loss of aldehydogenic material if the lysoplasmalogen (II) was the sole primary product; or to a mixture of (IV) and (III) with retention of the aldehydogenic material in (III) if the primary product was a mixture of both forms. Lysoplasmalogen was prepared from a mixture of lecithin and choline plasmalogen by (a) mild alkaline hydrolysis, which was accompanied by some loss of aldehyde as before, and by enzymic hydrolysis (b) with snake venom in which there was no loss of aldehyde. After hydrogenation there was a complete loss of aldehydogenic material in both (a) and (b), indicating that (II) is the sole primary product.

On treatment of (a) and (b) with 90% acetic acid at 50°, the usual analytical conditions in which the aldehyde moiety of (I) is completely hydrolysed, only 85% of the aldehyde was liberated, the remainder being converted into (III) which was isolated and characterised. Ethanolamine lysoplasmalogen and the cyclic acetal of glycerylphosphorylethanolamine was obtained in a similar way by alkaline hydrolysis of the kephalin fraction of ox heart, and subsequent acid hydrolysis.

There are two possible explanations of our early conclusions that the cyclic acetal compound was formed during alkaline hydrolysis. In one instance the material obtained by alkaline hydrolysis had inadvertantly been overneutralized with HCl to pH 4 before being readjusted to pH 7. Also in the early experiments the material after alkaline hydrolysis was purified on silicic acid columns. Evidence has been obtained that cyclisation of lysoplasmalogen can take place in these circumstances.

In the course of the isolation procedure, it became apparent that another phospholipid, very stable both to acid and alkali, was present in the hydrolysis products of the lecithin fractions, and a similar stable compound was found in the

kephalin products. These compounds were tentatively identified as glyceryl ethers of phosphorylcholine and phosphorylethanolamine[3]. The question arose whether the glyceryl ether might be an artifact arising from some side reaction in the deacylation of the plasmalogen such as was originally thought might account for the formation of the cyclic acetal compound. A mixed kephalin/kephalin plasmalogen fraction was therefore treated first with 90% acetic acid, which removed aldehyde completely from the kephalin plasmalogen with the formation of lysokephalin. The kephalin and lysokephalin were separated chromatographically and deacylated by treatment with mild alkali. All the lipid P in the lysokephalin fraction became water-soluble, but the kephalin fraction yielded some acid- and alkali-stable lipid P amounting to about 2% of the original lipid P. This stable phospholipid was identical to the material (glyceryl ether) found together with the cyclic acetal phospholipid after hydrolysis of (I) first with alkali and then with acid. The isolation from the kephalin, not the lysokephalin fraction indicates that the glyceryl ethers occur as acyl derivatives, but their similarity to the diacyl phospholipids makes their detection in this fraction very difficult by the usual analytical methods, owing to the small amounts present.

If the tissue phospholipids contain a large percentage of plasmalogens the determination of phospholipid composition by the analysis of hydrolysis products is subject to the following possible errors; (a) the alkaline hydrolysis produces a loss of total aldehydogenic material due to a partial splitting of the α,β-unsaturated ether bonding and gives rise to some glycerylphosphoryl-choline or glycerylphosphoryl-ethanolamine, which would be attributed to lecithin and phosphatidylethanolamine; (b) the partial cyclisation of the straight chain lysoplasmalogen during the subsequent mild acid hydrolysis yields some acid-stable compound which would thus increase the yield of alkali- and acid-stable material usually taken as the amount of sphingomyelin present in the tissue.

EXPERIMENTAL

Analytical methods

Methods for total phosphorus, total nitrogen, amino nitrogen and fatty acid ester groups were those used by GRAY AND MACFARLANE[4]. The determination of the α,β-unsaturated ether double bond occurring in plasmalogens was carried out by the iodine method as described by RAPPORT AND FRANZL[5].

The estimation of aldehyde values was based on the methods described by GRAY AND MACFARLANE[4] but four variants of the hydrolysis conditions were used to obtain information on the acid-lability of the various aldehydogenic compounds. These were: (a) Hydrolysis with 90% acetic acid at 50° for 45 min. The liberated aldehydes were condensed with rosaniline (Feulgen reagent) at 0° for 20 min. (b) Hydrolysis in 90% acetic acid containing 0.5 N HCl and 0.0015 N $HgCl_2$ at 50° for 45 min followed by condensation with Feulgen reagent at 0° for 20 min. (c) Condensation with Feulgen reagent in the presence of 90% acetic acid for 20 min at 0° without previous hydrolysis at 50°. (d) Condensation with Feulgen reagent in presence of 90% acetic acid containing 0.5 N HCl and 0.0015 N $HgCl_2$ at 0° for 20 min without previous hydrolysis at 50°.

The amount of glyceryl ether in the lipid fractions stable to mild alkaline and mild acid hydrolysis was determined as the difference between total P and P soluble in 5% trichloroacetic acid after hydrolysis in 2 N HCl at 100° for 2 h.

Mild alkaline hydrolysis of choline plasmalogen

Hydrolysis in methanolic NaOH: A choline plasmalogen/lecithin fraction isolated from ox heart[4] (103 mg P, 50% plasmalogen) was dissolved in 464 ml of methanol, and the temperature of the solution was raised to 38°. 51 ml of 1 *N* methanolic NaOH (containing 5% water) was then added dropwise with stirring and the mixture was incubated at 38° for 15 min. The NaOH was neutralised by addition of ethyl formate[6]. The mixture was evaporated to dryness under reduced pressure, the residue was suspended in chloroform–methanol (4:1) and the insoluble material consisting mainly of sodium formate was removed by filtration. The filtrate (94 mg P = 91% of the initial total P) was evaporated to dryness and the residue resuspended in 15 ml of chloroform–ethanol–water (8:4:0.25). This solution was chromatographed on a column of cellulose (Whatman, standard grade, 50 g) in the same solvent, to remove glycerylphosphorylcholine.

After the recovery of the lipid material from the column, the glycerylphosphorylcholine could be eluted if necessary with ethanol followed by water. The recovered lipid (47.5 mg P) was recrystallised twice from 30 ml of *n*-hexane–acetone–chloroform (3:3:0.5) and washed with ether. This procedure efficiently removed the fatty acid esters formed during the initial hydrolysis. The final yield was 46.8 mg P (91% of the original plasmalogen P) with a ratio N:P, 1:1; $[\alpha]_D^{20}$ in chloroform–methanol (1:1, v/v) —9°. THANNHAUSER, BONCODDO AND SCHMIDT[7] found $[\alpha]_D^{22}$ + 6.25° and RAPPORT, LERNER, ALONZO AND FRANZL[8] $[\alpha]_D^{26}$ —8.6° for the straight chain ethanolamine lysoplasmalogen.

The material gave a molar ratio aldehyde: P of 0.94:1 by method B and 0.82:1 by method A; it was hydrolysed to the extent of 40% at 0° (method C), while intact plasmalogen was practically unattacked (2–3% hydrolysis). The ratio α,β-unsaturated ether bond:P varied from 0.85:1 to 0.95:1 for different samples of the isolated material. These differences were attributed to the difficulty of determining the end point exactly.

A sample of the isolated material was hydrolysed in 2 *N* HCl at 100° for 2 h. After hydrolysis and addition of an equal volume of 10% trichloroacetic acid the acid-soluble P was 94% of the total P.

A sample of the material (3.8 mg P) dissolved in 6 ml of 95% ethanol was hydrogenated in the presence of 30 mg of Adams catalyst at atmospheric pressure and room temperature. After 1 h the reduction was complete and no aldehyde was detected in the material after hydrolysis by method B.

The only aldehydogenic product of the alkaline hydrolysis of choline plasmalogen therefore is the straight chain lysoplasmalogen. The aldehyde: P and acid-soluble P values indicated the presence of another compound also stable to alkaline and acid hydrolysis.

Hydrolysis in 0.5 N aqueous NaOH. Lecithin (25 mg P, 44% plasmalogen) was suspended in 62.5 ml of water, at 38° and an equal volume of aqueous NaOH was added in dropwise with shaking. The mixture was incubated at 38° for 9 h and then neutralised by addition of 0.5 *N* HCl at 0°. After removal of water by evaporation *in vacuo* the residue was extracted and the products of hydrolysis were isolated by the same method as described for hydrolysis in methanolic NaOH. Yield, 9.2 mg P (37% of initial total P); α,β-unsaturated double bond:P, 0.91:1.

Formation of cyclic compound from choline lysoplasmalogen in 90% acetic acid

Lysoplasmalogen (4 mg P) obtained by hydrolysis of plasmalogen in 0.1 *N* methanolic NaOH was treated with 90% acetic acid (4 ml) at 50° for 45 min. Acetic acid was removed by evaporation under reduced pressure as a binary azeotrope with CCl_4. The unhydrolysable material (1 mg P) was purified by chromatography on cellulose followed by chromatography on silicic acid. The P-containing material was eluted as a single peak with chloroform–methanol 1:1 (v/v); the ratios aldehyde:N:P were 0.74:1:1. Paper chromatography on silicic acid impregnated Whatman No. 1 paper in diisobutylketone–acetic acid–water (40:20:3, v/v) at 2° (see ref. 9) for 36 h showed that two compounds were present. One had an R_F value similar to lysolecithin and contained P and choline but no aldehyde or ester. The properties of this material were consistent with it being a glyceryl ether of phosphorylcholine. The other compound had a slightly higher R_F value and contained aldehyde, P and choline.

The lysoplasmalogen from the aqueous alkaline hydrolysis of choline plasmalogen was also treated with 90% acetic acid for 45 min at 50°. Paper chromatography of the unhydrolysable lipid material, recovered by chromatography on cellulose and silicic acid columns as before, showed the presence of the same two compounds. A larger quantity (9.3 mg P; aldehyde: P, 0.82:1, method B) of material stable to 90% acetic acid was prepared and chromatographed on a column of basic alumina (20 g; M. Woelm-Eschwege, Grade I). The material eluted with chloroform–methanol (9:1, v/v) (Table I) had ratios of aldehyde:P, 0.33:1, 1:1, 0:1 and 0.05:1 by methods A, B, C and D respectively. $[\alpha]_D^{20}$ in chloroform–methanol (1:1, v/v) +2°. No α,β-unsaturated ether was detected.

TABLE I

SEPARATION OF THE CYCLIC ACETAL OF GLYCERYLPHOSPHORYLCHOLINE FROM GLYCERYL ETHER ON BASIC ALUMINA

Fraction numbers	*Solvent MeOH in $CHCl_3$ (%, v/v)*	*Total P (mg)*	*Aldehyde* (%)*	*Paper chromatography** No. of spots*
6–12	10	2.1	99	1
13–25	11	2.3	101	1
26–39	11	0.7	85	2
40–50	50	2.3	61	2

* % of total P present as aldehyde compound.
** Silicic acid paper, solvent diisobutylketone–acetic acid–water (40:20:3, v/v).

The analysis and properties of this compound are consistent with those expected of the cyclic acetal structure (III). The material eluted with higher concentrations of methanol in chloroform was a mixture of this compound and the presumed glyceryl ether.

Effect of glacial acetic acid on choline lysoplasmalogen

Lysoplasmalogen was incubated with glacial acetic acid for 2 h at 50°, and the acetic acid removed as a binary azeotrope with CCl_4. The relative aldehyde values of the product were 30, 100, 0 and 5 by methods A, B, C and D respectively; no α,β-unsaturated ether was detected, and it was chromatographically identical with the cyclic acetal isolated after hydrolysis of lysoplasmalogen in 90% acetic acid.

Samples of lysoplasmalogen (43 μg P) were heated at 50° for 45 min with 0.5 ml of 100%, 95%, 90%, 80% and 70% acetic acid solutions. The differences in the aldehyde values obtained by method B and method D indicated that almost complete cyclisation of the lysoplasmalogen occurred in glacial acetic acid, 30% in 95% acetic acid, and about 10–15% at the lower concentrations.

Mild alkaline hydrolysis of kephalin plasmalogen, hydrolysis in 0.1 N methanolic and 0.5 N aqueous NaOH

Samples of an ethanolamine plasmalogen/kephalin fraction isolated from ox heart, were hydrolysed in 0.1 *N* methanolic NaOH at 38° and 0.5 *N* aqueous NaOH at 50°. The products had a ratio α,β-unsaturated ether:P, 0.93:1 and were readily hydrolysable by 90% acetic acid (aldehyde method C) while only slight hydrolysis was obtained in these conditions with intact ethanolamine plasmalogen. After hydrogenation no aldehyde was detectable in the mixture. The only aldehydogenic product of mild alkaline hydrolysis of ethanolamine plasmalogen was therefore ethanolamine lysoplasmalogen (II).

Formation of cyclic compound from ethanolamine plasmalogen in 90% acetic acid

Ethanolamine lysoplasmalogen obtained as above was treated with 90% acetic acid at 50° and the unhydrolysable material was isolated by chromatography on cellulose and silicic acid columns. The ratio aldehyde:P was 0.62:1 and 65% of the total P was acid-soluble after hydrolysis in 2 *N* HCl for 2 h at 100°. Paper chromatography of the material showed the presence of two compounds, one of which had the same R_F value in diisobutylketone–acetic acid–water solvent at 2° as that of a glyceryl ether obtained by hydrogenation of ethanolamine lysoplasmalogen. The other compound had an R_F value identical with that of synthetic 2-aminoethyl, 2,3-O-hexadecylidene glycerol 1-phosphate. The relative aldehyde values obtained by methods A, B, C and D (30, 100, 0 and 5) were also consistent with the cyclic acetal structure (III). Mercuric chloride had apparently no effect on the rate of hydrolysis in 90% acetic acid at 50°. FEULGEN AND BERSIN[10] found that the hydrolysis of the cyclic acetal phospholipid isolated by them was catalysed by $HgCl_2$.

Cyclic acetal plasmalogen was conveniently prepared from ethanolamine lysoplasmalogen by incubation in glacial acetic acid at 50°

Hydrolysis of choline plasmalogen with snake venom

A lecithin/choline plasmalogen fraction (10 mg P, 38% aldehyde) was dissolved in anhydrous ether (167 ml) and 1.67 ml of a freshly prepared 0.5% solution of the dried venom of *Agkistrodon piscivorus piscivorus* was added. The mixture was vigorously shaken and kept overnight at room temperature. The ether was evaporated and the residue consisting of lysolecithin and lysoplasmalogen was dissolved in 95% ethanol (8 ml) and hydrogenated. After 2 h no aldehyde (method B) or α,β-unsaturated ether was detected. Thus the lysoplasmalogen (II) is the only aldehydogenic product of enzymic hydrolysis of choline plasmalogen.

Hydrolysis of the products of enzymic hydrolysis in methanolic NaOH, followed by 90% acetic acid

Lecithin (16.7 mg P, 52% aldehyde) was incubated with snake venom for 48 h. The ether was evaporated, and the residue was treated with 0.1 *N* methanolic NaOH

at 38°. After neutralisation and evaporation of the solvent, the residue was treated with 90% acetic acid for 45 min at 50°. The unhydrolysable material was isolated by chromatography on cellulose and silicic acid columns. 2.07 mg lipid P was recovered, aldehyde:P, 0.82:1. Paper chromatography showed the presence of two compounds, one with R_F value identical to that of the glyceryl ether (IV) prepared by reduction of the lysoplasmalogen (II) and the other with that of the cyclic acetal compound (III).

Comparison of hydrolysis of plasmalogens by alkali and by snake venom

A decrease of 12–18% in total aldehyde (method B) occurred during the mild alkaline hydrolysis of choline and ethanolamine plasmalogens while during hydrolysis with the snake venom the value of total aldehyde remained constant. The loss of total aldehyde occurred only during deacylation (Fig. 1), so it appears that the stability of the α,β-unsaturated ether bond in alkali may be decreased during chemical attack on an adjacent group. Any aldehyde split off would presumably be rapidly polymerised in these conditions.

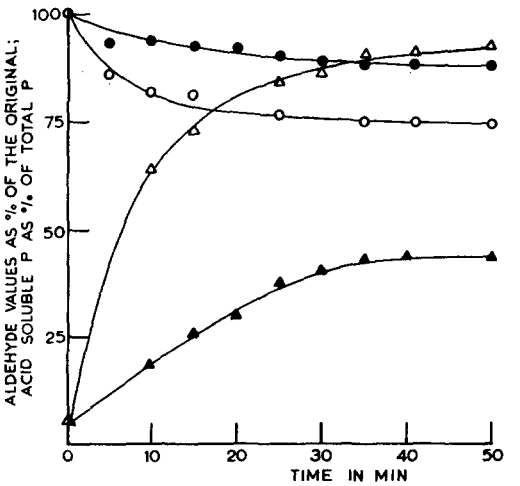


Fig. 1. Hydrolysis of ethanolamine plasmalogen with 0.1 *N* methanolic NaOH at 23°. Estimation of aldehyde by method A, ●—●; method B, ○—○; method C, ▲—▲. Acid-soluble P determined after treatment with 5% trichloroacetic acid for 30 min at 23°, △—△.

Occurrence of glyceryl ethers

Samples of an ox heart lecithin/choline plasmalogen fraction (1.4 mg P each, 50% plasmalogen) were hydrolysed (a) in 0.1 *N* methanolic NaOH followed by hydrolysis in 90% acetic acid at 50° for 45 min and (b) in 90% acetic acid at 50° followed by hydrolysis in 0.1 *N* methanolic NaOH. The recovered lipid material was chromatographed on silicic acid impregnated paper at 2° in diisobutyl ketone–acetic acid–water. Two compounds were present as products of procedure (a), one of which was chromatographically identical with the cyclic acetal phospholipid and the other with the glyceryl ether; only the glyceryl ether was present as the product of procedure (b).

A larger amount of a lecithin/choline plasmalogen fraction (100 mg P, 50% plasmalogen) was treated according to procedure (b) and the recovered lipid material was isolated by chromatography on cellulose and silicic acid columns. Yield of glyceryl ether, 3.2 mg P. The amount of glyceryl ether isolated from different batches of starting material, by procedure (a) (alkaline and acid hydrolysis) varied between 3–4% of the initial total P.

A kephalin fraction from ox heart (102 mg P, 42% plasmalogen) was hydrolysed in 90% acetic acid for 1.5 h at 50°. Lysokephalin and kephalin were separated by chromatography on silicic acid and subjected separately to alkaline hydrolysis. The unhydrolysable materials were purified by chromatography on cellulose and silicic acid columns. Almost all the glyceryl ether was present in the kephalin fraction, and only a trace was detected in the lysokephalin fraction. The yield was 2% of the initial total P.

In analytical conditions, the yield of material stable in 2 *N* HCl was the same whether alkaline hydrolysis was followed by 90% acetic acid hydrolysis or *vice versa*.

ACKNOWLEDGEMENTS

We wish to thank Professor T. MALKIN and Dr. T. H. BEVAN for the gift of 2-amino-ethyl, 2,3-O-hexadecylidene glycerol 1-phosphate. The support of this work by the British Empire Cancer Campaign is gratefully acknowledged.

REFERENCES

1 R. PIETRUSZKO AND G. M. GRAY, *Biochim. Biophys. Acta*, 44 (1960) 197.
2 J. B. DAVENPORT AND R. M. C. DAWSON, *Biochem. J.*, 79 (1961) 10P.
3 H. E. CARTER, D. B. SMITH AND D. N. JONES, *J. Biol. Chem.*, 232 (1958) 681.
4 G. M. GRAY AND M. G. MACFARLANE, *Biochem. J.*, 70 (1958) 409.
5 M. M. RAPPORT AND R. E. FRANZL, *J. Neurochem.*, 1 (1957) 303.
6 R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45.
7 S. J. THANNHAUSER, N. F. BONCODDO AND G. SCHMIDT, *J. Biol. Chem.*, 188 (1951) 417.
8 M. M. RAPPORT, B. LERNER, N. ALONZO AND R. FRANZL, *J. Biol. Chem.*, 225 (1957) 859.
9 G. V. MARINETTI AND E. STOTZ, *Biochim. Biophys. Acta*, 21 (1956) 168.
10 R. FEULGEN AND T. BERSIN, *Z. physiol. Chem. Hoppe-Seyler's*, 260 (1939) 217.