competitively inhibiting homoserine dehydrogenase. This is the most probable explanation for the previous observation that threonine inhibits the biosynthesis of bacteriochlorophyll and leads to an accumulation of porphyrins (Gibson *et al.* 1962*b*).

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The Formation of Cyclic Acetals during the Acid Hydrolysis of Lysoplasmalogens

BY J. B. DAVENPORT AND R. M. C. DAWSON

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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It has been recognized for some years that the fraction of tissue phospholipids stable to mild alkaline and acid treatment contained other phospholipids besides sphingomyelin (Brante, 1949; Dawson, 1954). In brain the amount of this non-sphingomyelin lipid was much reduced if the tissue lipids were initially precipitated with trichloroacetic acid, and the choline:phosphorus ratio of the fraction then approached unity (Dawson, 1954).

In a method developed by Dawson (1960) for the determination of individual phospholipids by successive chemical hydrolyses, the amount of non-sphingomyelin phospholipid in the fraction stable to mild hydrolysis by both alkali and acid was roughly proportional to the amount of plasmalogen present in the original phospholipid mixture. This suggested that such unknown phospholipid material might be formed from the plasmalogens during either the mild alkaline or the acid hydrolyses. The present paper describes the chemical characterization of one of the unknown phospholipids as a cyclic acetal of glycerylphosphorylethanolamine and presents evidence showing that it, together with free glycerylphosphorylethanolamine, is formed from ethanolamine lysoplasmalogen (alkylvinyl ether of glycerylphosphorylethanolamine) during mild acid hydrolysis. It also shows that the formation of the cyclic acetal can be considerably reduced by the addition of Hg^{2+} ions to the hydrolysing reagent. These results have been briefly reported by Davenport & Dawson (1961). Similar conclusions have also been reached by Pietruszko & Gray (1962).

EXPERIMENTAL

Isolation of the ox-heart-muscle phospholipids stable to mild acid and alkaline hydrolysis

Ventricular and auricular muscle (1200 g.) from ox heart was trimmed to remove connective and adipose tissue, and homogenized with 2 l. of chloroform–methanol (2:1, v/v). The extraction was continued overnight at room temperature and the extract then filtered through glass wool. The filtrate, consisting of two phases, was boiled for 5 min. and again filtered to remove precipitated material. The tissue residue from the original extraction was re-extracted twice with 500 ml. of hot chloroform. The total extracts were combined, and the top methanol–water layer was separated and re-extracted with 1 l. of chloroform; this extract was combined with the original chloroform layer. The combined chloroform solution was washed with 1·5 l. of 0·9% NaCl and then filtered through several layers of filter paper. This solution contained 43·2 g. of lipid which had 1·6% of phosphorus and 0·9% of nitrogen (N:P ratio 1·2).

The chloroform solution was evaporated to approx. 500 ml., 400 ml. of methanol and 100 ml. of 10% (w/v) NaOH were added and the mixture was incubated at 37° for 2 hr. It was then evaporated almost to dryness, 500 ml. of N-HCl and 1 l. of chloroform–methanol (1:1, v/v) were added and the mixture was again incubated at 37° for 1 hr. with continuous shaking. The two phases were separated and the lower layer was washed twice with 0·9% NaCl and evaporated to about 50 ml. Acetone (300 ml.) was added and the precipitate that formed during 2 hr. at −15° was filtered off rapidly through sintered glass. The filtrate was evaporated to dryness, the residue was dissolved in a further 100 ml. of acetone, and this solution was cooled for 2 hr. at −15° and filtered. The final mother liquors were evaporated to dryness and the residue was dissolved in a little chloroform and chromatographed on a silicic acid (Mallinckrodt) column (2 cm. diam. × 20 cm. long). Material eluted with chloroform was rejected and the column was washed with 100 ml. of chloroform–methanol (1:1, v/v) and 100 ml. of the same solvent containing 12% (v/v) of water. These two eluates were evaporated to dryness and added to the material insoluble in acetone at −15°. The final alkali- and acid-stable, acetone-insoluble fraction weighed 8·92 g., and contained 0·67% of phosphorus and 0·45% of nitrogen (N:P ratio 1·5). Preliminary chromatography on silicic acid showed that it contained considerable amounts of cerebroside-like lipids. It was therefore crystallized from 20 ml. of pyridine at 4°. This product weighed 1·88 g. and contained 2·0% of phosphorus (63% of the phosphorus in the acetone-insoluble material).

Silicic acid chromatography. The alkali- and acid-stable phospholipids which were insoluble in pyridine were subjected to silicic acid chromatography (40 g. of Mallinckrodt silicic acid from which the fines had been removed by washing with methanol and which had been reactivated by heating at 110° for 24 hr.) with a gradient of increasing concentration of methanol in chloroform.

Preparation of other lipids. Ethanolamine lysoplasmalogen was prepared from an ethanolamine-rich phospholipid fraction of ox brain by the method of Rapport, Lerner, Alonzo & Franzl (1957). Sheep-heart and ox-brain phospholipids were extracted and washed by the method of Folch, Lees & Sloane-Stanley (1957).

Chloroform–isobutanol solutions of alkali-stable phospholipid of ox heart and of ox brain were prepared by the method of Dawson (1960). They were washed with an equal volume of 0·9% NaCl to ensure that any remaining traces of water-soluble phosphorus were removed.

Hydrogenation of phospholipids followed by examination of the products of mild acid and mercuric chloride hydrolysis

To investigate whether cyclic acetal was present in the lipid mixture after alkaline hydrolysis, the mixture was hydrogenated to prevent further formation of the cyclic acetal from the alkylvinyl ether group on subsequent acid hydrolysis. The alkali-stable phospholipids from ox brain (5·3 mg. of original lipid P) were hydrogenated in chloroform solution with 20 mg. of Adams platinum catalyst until hydrogen uptake was negligible. The hydrogenated material was mildly hydrolysed with trichloroacetic acid (Dawson, 1960) and the water-soluble phosphate esters released were stored at 0° for chromatography.

The alkali- and acid-stable hydrogenated phospholipids were hydrolysed with 1% (w/v) $HgCl_2$ at 100° for 1 hr. by the method of Thannhauser, Boncoddo & Schmidt (1951*a*). The $HgCl_2$ was removed with silver oxide and hydrogen sulphide from the water-soluble phosphate esters released so that they could be examined by paper chromatography.

An identical sample of brain lipid was first hydrogenated as described above and then submitted to successive hydrolyses with alkali, trichloroacetic acid and $HgCl_2$ in an identical manner to that of the first sample.

Samples of each of the hydrolysis samples were submitted to unidimensional descending paper chromatography with the phenol–acetic acid–ethanol–water solvent of Dawson (1960). After the phosphorus-containing spots had been located with the acid molybdate spray of Hanes & Isherwood (1949) they were cut out and the phosphorus was determined.

Analyses. Phosphorus was determined by the method of Fiske & Subbarow (1925). Simultaneous determinations of nitrogen and phosphorus were carried out on a Kjeldahl digest (Lang, 1958). A sample containing 0·5–2·0 μg. atoms of N and P was digested with 0·5 ml. of digestion mixture at 310° for 4 hr. The digest was made up to 10 ml. with water; 3·0 ml. was taken for determination of nitrogen by nesslerization (Lang, 1958) and the remaining 7·0 ml. for determination of phosphorus by the method of Fiske & Subbarow (1925). Pure ovolecithin was used as a standard. Glycerol was determined by the method of Hanahan & Olley (1958).

RESULTS

Separation of alkali- and acid-stable phospholipids on silicic acid

The results of chromatography of the acid- and alkali-stable phospholipids of ox heart on silicic acid are presented in Fig. 1. Four main phosphorus- and nitrogen-containing peaks were obvious, but the tubes were combined in five fractions and analysed as shown in Table 1.

The main phosphorus-containing peak (D) appeared from its N:P ratio to be largely sphingo-

myelin. This was confirmed by analysis of the material after rechromatography on alumina according to Rhodes & Lea (1957) (Found: P, 3·68%; N:P ratio, 1·95. Calc. for *N*-lignoceryl-sphingosylphosphorylcholine: P, 3·7%; N:P ratio, 2). After this purified material had been split with methanolic hydrochloric acid, the phosphate esters detected by two-dimensional chromatography were phosphorylcholine and sphingosylphosphorylcholine, which is consistent with its identification as sphingomyelin (Dawson, 1958). The peak E forms a shoulder on the main sphingomyelin peak and analysis of the fraction suggests it contains mainly sphingomyelin together with an unknown ninhydrin-reacting substance of N:P ratio 1.

Fraction A was shown by further chromatography on silicic acid to be a mixture. After hydrolysis with methanolic 1·85N-hydrochloric acid for 4 hr. at 105°, 29% of the phosphorus remained ether-soluble. It was not further examined.

The second most prominent phosphorus-containing peak (B) was crystallized from hot ethanol. The lipid gave strong ninhydrin and Schiff's reactions and after acid hydrolysis (2N-hydrochloric acid for 2 hr. at 100°) glycerophosphate and ethanolamine could be detected in the hydrolysate by paper chromatography. After hydrolysis in 1% mercuric chloride at 100°, glycerylphosphorylethanolamine was detected by paper chromatography. The lipid was analysed (Found: P, 6·3; N, 2·9%; N:P ratio,

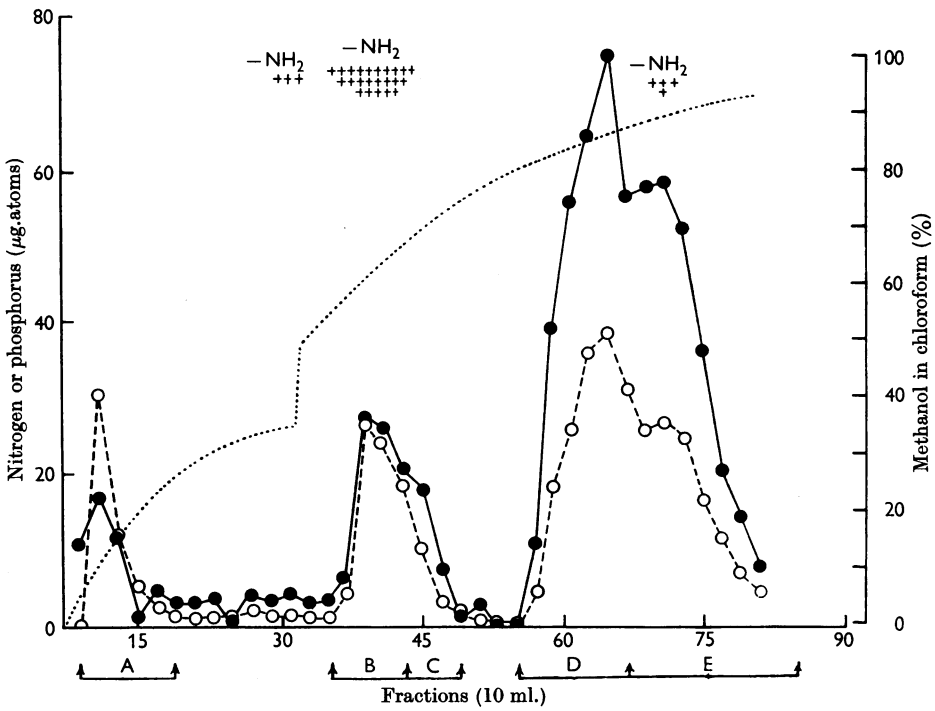


Fig. 1. Gradient elution of purified alkali- and acid-stable phospholipids from silicic acid (chloroform with increasing methanol concentrations). Experimental details are given in the text. ●—●, N; ○---○, P; ·····, methanol in chloroform gradient. —NH_2, Material reacting with ninhydrin (intensity indicated by plus signs).

Table 1. *Analysis of the peaks from silicic acid chromatography of phospholipids stable to mild alkaline and acid hydrolyses*

Fractions A–E and the tube numbers are those of Fig. 1. Experimental details are given in the text.

Fraction	Tube no.	Wt. (mg.)	P (%)	N (%)	N:P ratio
A	9–19	67	4·4	1·1	0·55
B	37–43	42	6·2	2·8	1·00
C	44–49	—	—	—	1·30
D	55–67	186	3·5	2·8	1·77
E	68–85	144	3·9	2·8	1·60

1·02. The cyclic acetal, 2-aminoethyl 2,3-*O*-octadecylidene glycerophosphate, requires: P, 6·65; N, 3·0%; N:P ratio, 1·01). When the unknown was examined in the infrared its spectrum showed bands at 3400, 2920, 2850, 2150, 1720, 1643, 1550, 1473, 1415, 1376, 1245, 1223, 1143, 1127, 1095, 1077, 1029, 1005, 962, 917, 875, 827, 760 and 721 cm.$^{-1}$. Of three synthetic cyclic acetals, kindly supplied by Dr T. H. Bevan, Department of Organic Chemistry, University of Bristol, namely the 2-aminoethyl esters of *cis*- and *trans*-2,3-*O*-hexadecylidene-1-glycerophosphoric acid and 1,3-*O*-hexadecylidene-2-glycerophosphoric acid, the spectrum of the unknown compound was virtually identical with the first.

When the lipid was hydrolysed with 2N-hydrochloric acid at 100° for 2 hr. about 10% of the phosphorus remained soluble in chloroform. Since the phosphorus of synthetic cylic acetal became completely water-soluble under the same conditions, it is probable that the isolated cyclic acetal is contaminated with glyceryl ether phospholipids of the type originally described by Carter, Smith & Jones (1958).

Fraction C, which adjoined the cyclic acetal peak, had a N:P ratio greater than 1·0, suggesting that it contained a nitrogen-containing substance as well as the cyclic acetal. It was not investigated further but the contaminant was probably cerebroside not removed by the pyridine treatment. This is known to be eluted from silicic acid at about this point in a methanol–chloroform gradient (Weiss, 1956).

Stability of cyclic acetal and lysoplasmalogen to mild acid hydrolysis

When solutions of the isolated and synthetic cyclic acetal were shaken with 10% trichloroacetic acid at 37° (Dawson, 1960) there was no liberation of water-soluble phosphorus. Nor was any produced when the trichloroacetic acid was made 5 mM with respect to mercuric chloride. On the other hand ethanolamine lysoplasmalogen broke down almost completely under such hydrolytic conditions, liberating water-soluble phosphorus.

Conditions for the formation of cyclic acetal from lysoplasmalogen

The question arose whether the cyclic acetal isolated was originally present in the living tissue or whether it had been formed during the process of isolation. The following observations indicate that it can be formed during the acid hydrolysis of lysoplasmalogen. This latter compound is formed from naturally occurring plasmalogen by alkaline hydrolysis (Rapport *et al.* 1957).

Alkali treatment. Samples of ox-brain lysoplasmalogen were incubated with alkali for periods of 5 and 75 min., the alkali was removed and the phospholipid then hydrolysed by shaking it with 10% trichloroacetic acid at 37° (Dawson, 1960). On distributing the hydrolysate between chloroform and water the percentage of phosphorus in the hydrolysate which remained chloroform-soluble did not increase with the longer period of alkali treatment (Table 2). Since both the isolated and synthetic cyclic acetals were completely stable to acid hydrolysis under these conditions, these results suggested that cyclic acetal was not produced by prolonged treatment of lysoplasmalogen with alkali.

The possibility remained, however, that cyclic acetal could be formed from intact plasmalogen at the moment of deacylation by alkali. A sample of brain phospholipid was therefore saponified both before and after catalytic hydrogenation as described in the Experimental section. Such a hydrogenation would be expected to saturate the vinyl ether linkage of natural plasmalogen and of lysoplasmalogen formed therefrom by saponification, and to remove any chance of cyclic acetal formation during subsequent hydrolyses. The resulting lipid was then examined for the presence of cyclic acetal by comparing the release of water-soluble phosphorus during mild acid hydrolysis (Dawson, 1960) and during mercuric chloride hydrolysis at 100° [which would break down the cyclic acetal (Thannhauser *et al.* 1951*a*)]. No water-soluble phosphorus was released by either treatment; this showed that hydrogenation had been complete and that no cyclic acetal was formed during the treatment of a phospholipid mixture rich in plasmalogen with alkali at 37°.

Acid treatment and the effect of mercuric ions. Since mercuric chloride is known to hydrolyse the plasmalogens catalytically (see, for example, Norton, 1959) the effect of Hg^{2+} ions on the acid hydrolysis of lysoplasmalogens was next investigated. A sample of alkali-stable phospholipids

Table 2. *Distribution of phosphorus between chloroform and water after successive alkaline and acid treatment of ox-brain lysoplasmalogen*

The ox-brain lysoplasmalogen was treated with alkali (methanolic 0·25N-NaOH at 37°) for the periods stated and then with 10% trichloroacetic acid for 30 min. at 37° (Dawson, 1960). The values are the means of four determinations. Experimental details are given in the text.

Period of alkali treatment (min.)	P in aqueous phase (μg.)	P in chloroform phase (μg.)
5	47·8	25·8
75	46·9	22·0

Table 3. *Effect of mercuric ions on the mild acid hydrolysis of alkali-stable phospholipids of heart*

The alkali-stable phospholipids were hydrolysed with 10% (w/v) trichloroacetic acid with or without $HgCl_2$ (5 mM) at 37° and then treated with methanolic HCl at 105° according to Dawson (1960). The hydrolysates were examined by paper chromatography of the water-soluble phosphate esters (Dawson, Hemington & Davenport, 1962). Experimental details are given in the text.

Compound formed		Initial hydrolysis by trichloroacetic acid (μg. of P)	Initial hydrolysis by trichloroacetic acid + $HgCl_2$ (μg. of P)
Glycerylphosphorylethanolamine	during the trichloroacetic acid hydrolysis	38·0	44·9
Glycerylphosphorylcholine	during the trichloroacetic acid hydrolysis	45·6	57·0
Unknown X (resulting from cyclic acetal hydrolysis)	during the methanolic HCl hydrolysis	18·5	4·2
Phosphorylcholine + sphingosylphosphorylcholine	during the methanolic HCl hydrolysis	15·2	15·0

from heart was hydrolysed with 10% (w/v) trichloroacetic acid at 37° both with and without mercuric chloride (5 mM), and the water-soluble phosphate esters were examined by paper chromatography (Dawson, 1960). The phospholipids stable to this mild acid hydrolysis were split with methanolic hydrochloric acid and again examined by paper chromatography (Dawson, 1960). Table 3 shows that the presence of Hg^{2+} ions had appreciably increased the amount of glycerylphosphorylethanolamine and glycerylphosphorylcholine formed during the mild acid hydrolysis. At the same time, on chromatography of the products formed from the stable phospholipids on heating with methanolic hydrochloric acid, there was a corresponding decrease in the phosphorus spot formed from cyclic acetal whereas the phosphorylcholine plus sphingosylphosphorylcholine spot (from sphingomyelin) did not change (Table 3). As the isolated cyclic acetal was completely stable to hydrolysis with trichloroacetic acid at 37°, either in the presence or absence of Hg^{2+} ions, these results indicate that the presence of Hg^{2+} ions during the hydrolysis of the lysoplasmalogens prevented the formation of cyclic acetal and increased the yield of glycerylphosphoryl bases. This was confirmed in the experiment shown in Table 4, in which the alkali-stable phospholipids from brain were hydrolysed in the presence of acid both with and without added Hg^{2+} ions. The greatest liberation of water-soluble phosphorus was obtained when Hg^{2+} ions were present from the beginning of the hydrolysis. When Hg^{2+} ions were added to the system after 30 min. and hydrolysis was continued for a further 30 min. the release of water-soluble phosphorus was no greater than when no Hg^{2+} ions were present throughout the two hydrolysis periods.

The release of glycerylphosphoryl bases from alkali- and acid-stable phospholipids from brain or heart was not further increased by increasing the Hg^{2+} ion concentration, omitting the acid and adding it later, or by substituting mercuric acetate

Table 4. *Successive acid hydrolysis of the alkali-stable phospholipids of brain with and without mercuric ions*

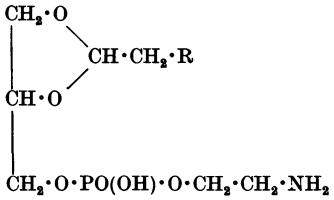
The hydrolysis was conducted by the two-phase technique described by Dawson (1960). The hydrolysis reagents were: A, 10% (w/v) trichloroacetic acid; B, 10% (w/v) trichloroacetic acid containing $HgCl_2$ (15 mM). The chloroform–isobutanol solution of the alkali-stable phospholipids (1 ml.) was shaken for 30 min. at 37° with 0·4 ml. of reagent A or B. A further 0·4 ml. of reagent (A or B) was then added and the hydrolysis continued for another 30 min. period at 37°. Experimental details are given in the text.

Hydrolysis reagents added		Water-soluble P released (μg.)
First 30 min. period	Second 30 min. period	
A	A	53·0, 51·3
B	A	60·2, 60·2
A	B	53·0, 52·4

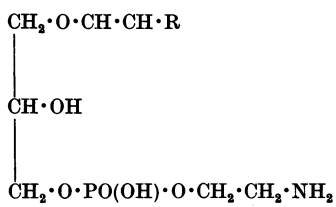
for the mercuric chloride. The mixture obtained by treatment of the remaining phospholipids with methanolic hydrochloric acid still showed on paper chromatography the presence of a small spot which corresponded to cyclic acetal. Further, traces of glycerylphosphoryl base derivatives could still be detected by chromatography after these remaining phospholipids had been hydrolysed for 1 hr. at 100° with 1% mercuric chloride. There were also considerable amounts of phosphorylcholine derived from sphingomyelin in the latter hydrolysates.

DISCUSSION

In the past the preparation of so-called 'plasmalogen' has always involved the treatment of the tissue phospholipids with alkali to decompose the diacylated phospholipids. The compounds isolated were until recently thought to have the cyclic acetal structure (I) (e.g. Feulgen & Bersin, 1939; Thannhauser *et al.* 1951*a*, *b*). However, it was conclusively demonstrated by Rapport *et al.* (1957)



(I)



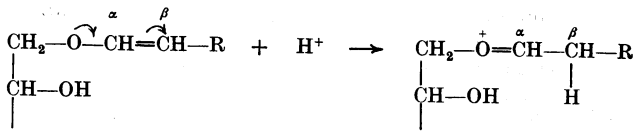
(II)

that the ethanolamine lysoplasmalogen which they isolated from muscle phospholipids after alkaline treatment had a vinyl ether structure (II). This structure is now generally accepted as being the deacylated form of the naturally occurring plasmalogen, although there is still some doubt whether the unsaturated ether is combined through the α or β position of the glycerol (Marinetti, Erbland & Stotz, 1958). The choline-containing analogue first isolated in crystalline form by Hartree & Mann (1960) from spermatozoa also apparently possesses the vinyl ether structure.

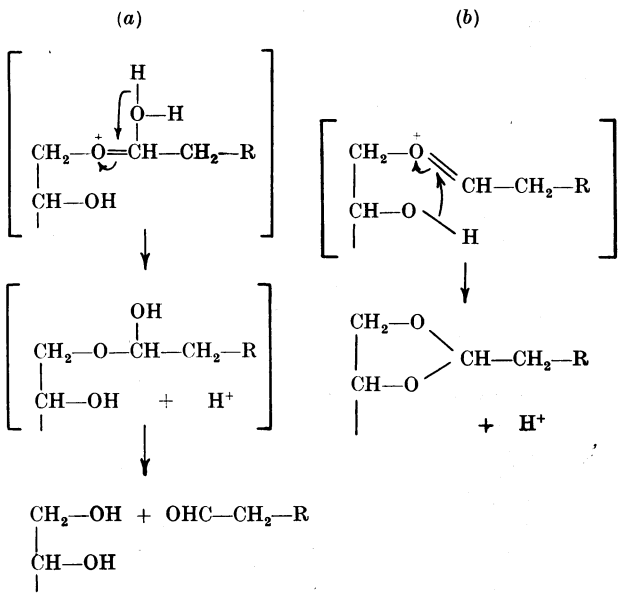
From the results presented by Feulgen & Bersin (1939) and Thannhauser *et al.* (1951*a*, *b*) it is not possible to decide with certainty the structure of the plasmalogen which they isolated, although the absence of unsaturation reported by the latter group of workers would suggest that it may have had the cyclic acetal rather than the vinyl ether structure.

In the present work the cyclic acetal of glycerylphosphorylethanolamine has been isolated from the ox-heart phospholipids which are stable to mild alkaline and acid hydrolysis. The major portion of this (and probably all) was, however, not originally present in the tissue but was formed from lysoplasmalogen [presumably of structure (II)] on mild acid treatment. No evidence was obtained that the cyclic acetal of glycerylphosphorylethanolamine could be formed from lysoplasmalogen by treatment with alkali. Such a transformation was originally reported for the choline analogue by Pietruszko & Gray (1960), but later hydrogenation experiments similar to those reported here have suggested that the original results require a different interpretation (Pietruszko & Gray, 1962).

In the lysoplasmalogen structure an electromeric shift through the unsaturated bond would occur rendering the β carbon a centre for attack by



Scheme 1.



Scheme 2.

an electrophilic group such as a H^+ ion but not by nucleophilic reagents such as an OH^- ion (Scheme 1). This would give rise to the extreme canonical form on the right-hand side of the equation. This would then be capable of reacting with water with the formation of a hemiacetal which would spontaneously break down to give free aldehyde (*a*) or it could react with the neighbouring hydroxyl group of the glycerol to form a cyclic acetal (*b*) (Scheme 2). When the initial electrophilic reagent is a Hg^{2+} ion, reaction with the neighbouring hydroxyl group is apparently suppressed.

Our results are in substantial agreement with those of Schmidt *et al.* (1959), who showed that more water-soluble phosphorus was liberated from alkali-stable brain phospholipids when these were hydrolysed with mercuric chloride solution rather than with acid. It would seem that when the attacking electrophilic group is a Hg^{2+} ion rather than a H^+ ion the intermediate (right-hand side of Scheme 1) reacts much more rapidly with water than it does with the neighbouring vacant hydroxyl group.

Even after the hydrolysis of the alkali-stable phospholipids in the presence of Hg^{2+} ions a small

amount of cyclic acetal is found in the remaining lipids. Whether this is originally present in the tissue or is still formed as an artifact from lysoplasmalogen cannot be deduced from the present evidence. The analytical results of Rapport & Lerner (1959), who compared the amount of iodine taken up by the unsaturated α,β ether grouping with the total aldehyde measured as *p*-nitrophenylhydrazone, suggest that very little cyclic acetal can be present in mammalian tissues, although more may be present in marine invertebrates (Rapport & Alonzo, 1960).

The silicic acid chromatography of the present preparations of the alkali- and acid-stable phospholipids gave no peak which could be designated as the cyclic acetal of glycerylphosphorylcholine. Thus, none of the fractions gave glycerylphosphorylcholine on hydrolysis with mercuric chloride at 100° (Thannhauser *et al.* 1951*a*). The results presented in Table 3 strongly suggest, however, that such a cyclic acetal is formed on acid hydrolysis of the alkali-stable phospholipids since the amount of glycerylphosphorylcholine produced was appreciably increased when Hg^{2+} ions were added to the hydrolysing medium. It is possible that the cyclic acetal of glycerylphosphorylcholine was lost during the solvent fractionations to remove cerebrosides or that it was not eluted from the column.

The present results therefore show that the non-sphingomyelin phospholipids present in the alkali- and acid-stable phospholipid fraction are largely cyclic acetal plasmalogens formed as artifacts during the acid treatment. In certain tissues small amounts of glyceryl ether phospholipids would also be present in this fraction (Svennerholm & Thorin, 1960; Ansell & Spanner, 1961) and it is probable that other minor components will eventually be identified.

SUMMARY

1. The cyclic acetal of glycerylphosphorylethanolamine has been isolated from the ox-heart phospholipid fraction stable to both alkaline and acid hydrolysis.

2. Evidence is presented that this is formed by the acid hydrolysis of ethanolamine lysoplasmalogen [α-(alkylvinyl ether) of glycerylphosphorylethanolamine] which is itself known to be produced when natural plasmalogen is treated with alkali. Glycerylphosphorylethanolamine is also formed from ethanolamine lysoplasmalogen during the acid hydrolysis.

3. Addition of Hg^{2+} ions to the acid greatly reduced the proportion of cyclic acetal produced in the reaction.

4. No evidence could be obtained that the cyclic acetal form of plasmalogen was formed on alkaline hydrolysis of naturally-occurring plasmalogen.

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