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The Reaction of Phosphoglycolipids and other Lipids with Hydrofluoric Acid

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1. The use of HF as a dephosphorylating reagent for phospholipids was examined. 2. Hydrolysis of phosphatidylethanolamine at 0°C for 24h with 60% HF gives a good yield of diglyceride. Under similar conditions phosphatidyldiglucosyl diglyceride gives diglyceride and diglucosyl diglyceride. 3. The glycolipid is also obtained from hydrolysis of glycerylphosphoryldiglucosyl diglyceride. No lyso derivative of the glycolipid could be detected and the glycosidic linkage was also stable. 4. Triglycerides, unsaturated and cyclopropane fatty acids were unaffected by the reagent. 5. 1,2-Diglycerides and 1,3-diglycerides were partially isomerized and also gave small amounts of free fatty acid and monoglyceride. 6. Monoglycerides underwent extensive rearrangement to form 1,2- and 1,3-diglycerides. 7. Lysophosphatidylethanolamine also gave 1,2- and 1,3-diglycerides as well as monoglycerides. 8. The application of this procedure to the structure determination of various phosphoglycolipids is discussed.

Phosphorylated derivatives of glycosyl diglycerides, 'phosphoglycolipids', have been isolated from many bacteria (Fischer, 1970; Shaw *et al.*, 1970; Ambron & Pieringer, 1971; Shaw & Stead, 1972). The phosphoglycolipid isolated from *Acholeplasma laidlawii* contained two acyl residues (Shaw *et al.*, 1970), whereas the lipid isolated from several streptococci appeared to contain three or four acyl residues (Fischer, 1970; Ambron & Pieringer, 1971). Several lines of evidence indicated that these phosphoglycolipids were either glycerylphosphoryl or phosphatidyl derivatives of diglucosyl diglycerides, but clearly a rigorous proof of structure would require the isolation of the intact glycolipid from the phosphoglycolipids and the precise location of any additional acyl residues. We have briefly reported the use of HF as a suitable reagent for these purposes (Shaw & Stead, 1972), and Shaw *et al.* (1972) have described the isolation of diglucosyl diglyceride from the glycerylphosphoryldiglucosyl diglyceride in *A. laidlawii*. HF has been used previously as a convenient reagent for the dephosphorylation of certain polymers containing phosphodiester linkages and Toon *et al.* (1972) have used it for investigating the structure of the lipoteichoic acid from *Streptococcus faecalis* in which the covalently bound lipid moiety may be structurally related to the phosphoglycolipids present in the lipids of this organism. We have therefore undertaken a detailed examination of the reaction of HF with phosphoglycolipids and other lipids.

Materials and Methods

Materials

'Mallinckrodt 100 mesh' silicic acid was obtained from Kodak Limited, Kirkby, Lancs., U.K. Phosphatidyldiglucosyl diglyceride and diglucosyl diglyceride were isolated from the lipids of *S. faecalis* (Ambron & Pieringer, 1971); glycerylphosphoryldiglucosyl diglyceride was isolated from the lipids of *A. laidlawii* (Shaw *et al.*, 1968); fatty acid methyl esters were purchased from Applied Science Laboratories Inc., State College, Pa., U.S.A. A sample of the fatty acids from *Lactobacillus helveticus* was obtained by deacylation of the lipids. All other lipids were purchased from the Sigma (London) Chemical Co., London S.W.6, U.K.

Methods

Thin-layer chromatography. Glycolipids and phospholipids were analysed in the solvent system chloroform–methanol–water (65:25:4, by vol.; Wagner *et al.*, 1961) and detected with the periodate–Schiff reagents (Shaw, 1968) and the molybdate reagent (Dittmer & Lester, 1964). Neutral lipids were analysed in a two-dimensional system containing trimethyl borate to prevent isomerization [first direction: chloroform–ethanol–trimethyl borate (100:1:6, by vol.); second direction: benzene–ethyl acetate–trimethyl borate (100:20:7.2, by vol.); Pollack *et al.*,

1971]. Spots were detected by charring with H_2SO_4 and determined quantitatively in a Joyce–Loebl Chromoscan. Diglycerides were separated into individual molecular species by acetylation and argentation t.l.c. by the method of Renkonen (1965).

Gas–liquid chromatography. Fatty acid methyl esters were analysed on a Perkin–Elmer F11 gas chromatograph or a Pye series 104 gas chromatograph; both instruments were fitted with flame-ionization detectors. The column was packed with 15% (w/w) polyethylene glycol succinate on acid-washed Celite (80–100 mesh).

Silicic acid column chromatography. Mixtures of tri-, di- and mono-glycerides were separated on a column of silicic acid by elution with diethyl ether–light petroleum (b.p. 60–80°C) mixtures by the method of Hirsch & Ahrens (1958).

Analytical methods. Glucose was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956) and phosphorus by the method of Chen *et al.* (1956).

Hydrolysis with HF. The lipid (approx. 1 mg) was dissolved in a few drops of chloroform contained in a polytetrafluoroethylene tube and the solvent removed by evaporation under reduced pressure to leave a thin film of lipid across the bottom of the tube. The lipid was covered with 60% HF (0.2 ml) and kept at 0°C for the required length of time. To recover the lipid products from the reaction a mixture of ice-cold chloroform–water (1:1, v/v) was added and the chloroform layer rapidly separated. The latter was washed with $NaHCO_3$ solution (10%, w/v), water, and then dried over anhydrous Na_2SO_4. The products were then analysed where appropriate by t.l.c. or g.l.c.

Results

Neutral lipids

After exposure to HF for 24 h the relative amounts of the components of a mixture of fatty acid methyl esters remained unchanged. The mixture examined was obtained from the lipids of a lactobacillus, a genus which is known to contain both unsaturated and cyclopropane fatty acids; neither were affected under the conditions of the experiment. Triglycerides were also virtually unaffected and diglycerides underwent minor isomerization, but the most significant changes were observed with monoglycerides (Table 1). Both monopalmitin and mono-olein underwent extensive rearrangement to form diglycerides, but no triglycerides could be detected. This reaction was investigated further by analysing the diglycerides produced from an equimolar mixture of monopalmitin and mono-olein after treatment with HF. The diglyceride fraction was separated by silicic acid column chromatography, acetylated, and the resulting diglyceride acetates were separated into saturated, mono-unsaturated and di-unsaturated species by argentation t.l.c. The appropriate bands were eluted from the plate and the fatty acid components obtained by deacylation and identified by g.l.c. The saturated diglyceride contained only palmitic acid, the di-unsaturated diglyceride contained only oleic acid, and the mono-unsaturated diglyceride contained equimolar amounts of palmitic acid and oleic acid.

Glycolipids

Diglucosyl diglyceride was recovered virtually unchanged after treatment with HF for up to 24 h. No evidence for the production of lyso diglucosyl diglyceride (i.e. diglucosyl monoglyceride) or glucosyl diglyceride was obtained.

Phospholipids

The isolation of diglucosyl diglyceride from glycerylphosphoryldiglucosyl diglyceride by treatment with HF has been described previously (Shaw *et al.*, 1972). The same glycolipid has now been obtained from phosphatidyldiglucosyl diglyceride, together with 1,2- and 1,3-diglycerides, which represent over 95% of the neutral lipids formed in the reaction. The rate of hydrolysis was determined by

Table 1. *Analysis of the lipophilic products after treatment of various lipids with HF for 24 h*

Values given are percentages based on densitometry of t.l.c. after charring with H_2SO_4.

	Monoglyceride	1,2-Diglyceride	1,3-Diglyceride	Triglyceride	Fatty acid
Monopalmitin	30.5	37.5	28		3.5
1,2-Dipalmitin	0.5	77	17.5		4.5
1,3-Dipalmitin	0.5	15	76.5		7.0
Tripalmitin		0.5	1	95	3
Mono-olein	15.5	34	49.5		1
Diolein*	3	42.5	50.5		3.5
Triolein				100	
Phosphatidylethanolamine	2	87.5	6.5		3.5
Lysophosphatidylethanolamine	36.5	25.5	25		13

* Mixture of 1,3- and 1,2-diglycerides in ratio 91:9.

measuring the amount of organic phosphorus remaining in the chloroform layer after various time-intervals. After 24h 89% of the phosphatidyldiglucosyl diglyceride had been dephosphorylated. Under identical conditions 95% of phosphatidylethanolamine was hydrolysed. The neutral products from this reaction were also predominantly diglycerides (Table 1). Lysophosphatidylethanolamine was also hydrolysed and gave the same complex mixture of neutral lipids as obtained from monoglyceride (Table 1); diglycerides accounted for almost 50% of the neutral products.

Discussion

The non-enzymic cleavage of phosphatides to give diglycerides has been achieved previously both by acetolysis and by hydrolysis with HCl. We concluded, for several reasons, that such procedures would not be suitable for the isolation of lipophilic degradation products from phosphoglycolipids. Acetolysis, first described by Bevan *et al.* (1953), has been developed by Renkonen (1965) into a convenient method for the determination of molecular species in phosphatides. However, those phosphoglycolipids which do not contain the phosphatidyl group might not undergo acetolysis in the same manner as conventional phosphatides. Acetylation of sugar hydroxyl groups would be anticipated and subsequent isolation of any glycolipids produced would require preferential deacetylation; acetolysis of the glycosidic linkages could also not be excluded. The glycosidic linkages would also be susceptible to cleavage under the conditions required to obtain diglycerides from phosphatides by hydrolysis with HCl (Hanahan & Olley, 1958) and appreciable quantities of monoglyceride and free fatty acids are also produced by this method. HF was first used to dephosphorylate nucleic acids and has been used more recently to hydrolyse those teichoic acids which, because of their glycosyl substituents, are stable to alkali (Glaser & Burger, 1964; Archibald *et al.*, 1968). We have previously reported the use of this reagent for the isolation of diglucosyl diglyceride from the glycerylphosphoryldiglucosyl diglyceride of *A. laidlawii*. This phosphoglycolipid contains only two acyl residues and accordingly no other lipophilic products would be produced in the reaction. However, hydrolysis of a phosphatidyldiglucosyl diglyceride should yield both the glycolipid and diglycerides; a lyso derivative should yield a monoglyceride. Fig. 1 shows a schematic representation of the anticipated products from the hydrolysis of various phosphoglycolipids and their lyso derivatives. (The isomeric structures containing acyl residues esterified to the sugar residues have been omitted. Such phosphoglycolipids would also be expected to undergo hydrolysis with HF to yield acylated glycolipids, which should be easily recognized by their chromatographic properties.) Although many of these lipids have not been observed thus far, their occurrence is foreseeable and this method should enable their structures to be established. A necessary prerequisite, however, is to establish the stability, or otherwise, of the various products, monoglyceride, diglyceride, glycolipids, to further degradation by HF. Accordingly we have examined the reaction of these lipids with this reagent.

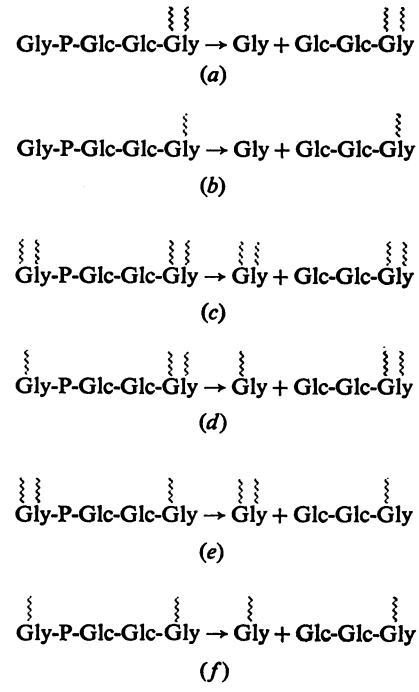

Fig. 1. *Schematic representation of the anticipated products from the reaction with HF of phosphoglycolipids with various degrees of acylation*

Although for convenience the phosphate residue is shown linked to the terminal sugar of a diglucosyl diglyceride, the reaction pathway should not be influenced by either the nature of the sugar or the location of the phosphate residue. (*a*) Glycerylphosphoryldiglucosyl diglyceride; (*b*) glycerylphosphoryldiglucosyl monoglyceride; (*c*) phosphatidyldiglucosyl diglyceride; (*d*) lysophosphatidyldiglucosyl diglyceride; (*e*) phosphatidyldiglucosyl monoglyceride; (*f*) lysophosphatidyldiglucosyl monoglyceride. Gly, monoglyceride; Gly, diglyceride.

A mixture of fatty acid methyl esters obtained from *L. helveticus* and known to contain saturated, unsaturated and cyclopropane fatty acids was recovered unchanged. In particular the relative proportions of the individual fatty acids was unchanged showing that no degradation of unsaturated or cyclopropane fatty acids had occurred. Diglucosyl diglyceride and

triglycerides were both stable, but 1,2- and 1,3-diglycerides underwent partial isomerization which probably occurred, not during the reaction itself, but during the isolation procedure as any prolongation of the latter increased the proportion of the two isomers. Somewhat unexpectedly monoglycerides underwent rearrangement to form considerable amounts of diglycerides. Both monopalmitin and mono-olein behaved similarly and a mixture of the two gave, besides dipalmitin and diolein, oleoyl palmitoyl diglyceride, indicative of a transesterification process.

Hydrolysis of phosphatidylethanolamine was 95% complete after 24h. Phosphatidyldiglucosyl diglyceride was hydrolysed more slowly, but both phospholipids gave diglyceride as the major lipophilic product. The behaviour of monoglycerides suggested that any lysophosphatide, which should initially give monoglyceride, would also give a complex mixture of neutral lipids and this was confirmed with chromatographically pure lysophosphatidylethanolamine. The major products were not monoglycerides but the same mixture of monoglycerides and diglycerides obtained from monoglyceride alone.

These results confirm the potentiality of HF as a dephosphorylating reagent for phospholipids and the method is particularly useful in those instances where acetolysis and hydrolysis with HCl is inappropriate. The isolation of diglycosyl diglycerides from phosphoglycolipids is an important facet in their structure determination and the concurrent formation of diglycerides provides additional evidence for the presence of a phosphatidyl group within the molecule. A comparison of the fatty acid components of the diglyceride with those of the glycolipid enables information on the distribution of particular fatty acids within the molecule to be obtained. However, in view of the rearrangement of monoglycerides, where the presence of lysophospholipids is suspected the results should be interpreted with caution.

Hydrolysis with HF of lipoteichoic acids isolated from several Gram-positive bacteria has demonstrated the presence of covalently bound glycolipids or phosphoglycolipids in these polymers (Toon *et al.*, 1972; Coley *et al.*, 1972). Lipoteichoic acids containing the teichoic acid linked through a phosphodiester linkage to either a diglycosyl diglyceride or a glyceryl-phosphoryldiglycosyl diglyceride would yield diglycosyl diglyceride as the sole lipophilic product. A lipoteichoic acid containing a phosphatidyldiglycosyl diglyceride would yield both diglyceride and diglycosyl diglyceride in equimolar proportions. Both products have been qualitatively identified from hydrolysis of the lipoteichoic acid in *S. faecalis* suggesting the presence of a phosphatidyldiglycosyl diglyceride (Toon *et al.*, 1972), but these products would also be given by a polymer containing a diglycosyl diglyceride and a phosphatidyl group at two separate locations within the molecule. Thus the identification of the lipophilic products produced by hydrolysis with HF cannot alone establish the nature of the lipid moiety in these polymers.

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