A New Bacterial Sphingophospholipid Containing 3-Aminopropane-1,2-diol

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1. A new sphingophospholipid has been isolated from the bacterial fraction of sheep rumen contents. 2. This new lipid has been characterized as a ceramide phosphate moiety esterified to 3-aminopropane-1,2-diol through the primary alcohol group. 3. Mass spectrometry has shown the intact lipid to contain a vicinal hydroxyl amino grouping, and oxidation with periodate converts it quantitatively into a new phospholipid which is probably ceramide phosphorylglycolaldehyde. 4. The sphingophospholipid contains a mixture of saturated long-chain bases mainly with branched-chain alkyl groups, which are typical of a microbial origin. 5. A Gram-negative bacterium isolated from rumen contents contains about 30% of the new lipid in its phospholipids when grown in pure culture.

Kemp & Dawson (1969) have described a new phosphoglyceride in rumen protozoa that has been characterized as *N*-(1-carboxyethyl)phosphatidylethanolamine. During these studies it became apparent that the bacterial fraction from the rumen contained a phospholipid with chromatographic properties different from those of previously described compounds. This phospholipid has now been isolated and characterized as a 1-(*N*-acylsphingosylphosphoryl)ester of 3-aminopropane-1,2-diol. Its bacterial origin has been confirmed by experiments with sheep with defaunated rumens and by the isolation in pure culture of a rumen micro-organism that contains appreciable quantities of the new sphingophospholipid in its structure.

Materials and Methods

Isolation of sphingophospholipid

The sheep used were Clun Forest possessing permanent rumen fistulae and fed on a diet of hay (800g)+oats (200g) daily. In some experiments the ciliate protozoa were removed from the rumen by treatment with detergent (Dawson & Kemp, 1969). The rumen contents were removed and strained through cheesecloth and the rumen liquor was centrifuged at 1500 rev./min for 2 min in an MSE Mistral 6L centrifuge (head 59563). The off-white pellet, consisting mainly of the larger protozoa, was rejected. The supernatant was centrifuged at 10000 g_{av} for 30 min in a Sorvall SL centrifuge. The pellet was extracted with 20 vol. of chloroform-methanol (2:1, v/v), the mixture being warmed to boiling point and

left overnight. The insoluble residue was filtered and re-extracted with a further 200 ml of the same solvent. The combined solvent extracts were shaken with 0.9% NaCl (0.2 vol.), and after the mixture had been left to stand the lower phase was evaporated to dryness. The residue was dissolved in the minimum amount of chloroform and methanolic 0.125 M-NaOH (1:1, v/v) (5% of original rumen fluid volume) and the mixture incubated for 1 h at 37°C. To the mixture was added 3 vol. of water, 2 vol. of ethanol and 4 vol. of chloroform. After the mixture had been shaken the lower phase was separated and evaporated to dryness.

The alkali-stable lipids dissolved in chloroform were loaded on to a silicic acid (Mallinckrodt CC4; 100–200 mesh) column [15 cm long $\times$ 1 cm diam.; poured in chloroform-methanol (1:1, v/v) and washed with chloroform] and successively eluted with 25 ml of chloroform-methanol (19:1, v/v), 50 ml of chloroform-methanol (4:1, v/v) and 75 ml of chloroform-methanol (3:2, v/v). The unknown sphingophospholipid plus ceramide phosphorylethanolamine were eluted with the last solvent. The mixture was run as a strip on a preparative t.l.c. plate (silica gel H, 250 μ m thick; activated for 1 h at 100°C) with chloroform-methanol-water-aq. NH_3 (sp.gr. 0.88) (110:50:5:8, by vol.) as solvent. After side marker lanes had been sprayed with ninhydrin (0.25% in acetone) the slowest-running strip (R_F 0.25 and usually brownish purple after ninhydrin reaction at 100°C for 5 min) was eluted by scraping off the silica gel and extracting it with chloroform-methanol (1:1, v/v). The ceramide phosphorylethanolamine (Dawson & Kemp, 1967, 1968) was also eluted when required for comparison. Rumen fluid (10 litres), free

of protozoa, yielded 0.75mg of P of the unknown phospholipid and 1.2mg of P of ceramide phosphorylethanolamine.

Isolation of water-soluble base from the unknown phospholipid

The unknown sphingophospholipid was hydrolysed in methanolic aq. HCl by the procedure of Gaver & Sweeley (1965). The hydrolysate was evaporated to dryness and partitioned between equal volumes of 6M-HCl and chloroform. The upper phase was hydrolysed for 48h at 100°C in a sealed tube. The HCl was removed as thoroughly as possible *in vacuo*, and the residue was applied as a strip to Whatman no. 1 paper washed in 2M-acetic acid and separated by ionophoresis (1h at 40V/cm) in pyridine-acetic acid-water (1:10:89, by vol.) buffer, pH 3.6 (Dawson *et al.*, 1962). The spraying of marker side lanes with ninhydrin located the position of a positively charged base. It had a mobility towards the cathode that was about 0.82 of that of ethanolamine. The base was chromatographically eluted from its strip with water. The eluate was adjusted to pH 2 and passed down a Zeo-Karb 225 sulphonated polystyrene cation-exchange column (20cm long × 1cm diam.). The column was copiously eluted with water and the base was then eluted with 5M-NH₃ and recovered by evaporation under reduced pressure.

Preparation of DL-3-aminopropane-1,2-diol

A 3g portion of 3-chloropropane-1,2-diol (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was heated with a threefold molar excess of aq. NH₃ (sp.gr. 0.88) for 60h at 50°C in a sealed tube. The reaction mixture was evaporated to dryness *in vacuo*, dissolved in 50ml of water, adjusted to pH 2 and loaded on to a Zeo-Karb 225 sulphonated polystyrene cation-exchange column (15cm long × 2cm diam.). The column was eluted with water to remove unchanged 3-chloropropane-1,2-diol and Cl⁻, and the 3-aminopropane-1,2-diol was removed by elution with 5M-NH₃. The eluate was evaporated in a rotary evaporator to remove water and NH₃, and the colourless liquid 3-aminopropane-1,2-diol was collected in good yield (Girdler Corp., 1937). The compound had a refractive index of n_D^{25} 1.4920 (water n_D^{25} was 1.3328) measured with an Abbe refractometer. Schreyer (1951) gives a n_D^{25} of 1.4935 for DL-3-aminopropane-1,2-diol prepared by another method.

Mass spectra

Mass spectra were recorded on an Associated Electrical Industries MS902 mass spectrometer, fitted with a direct-insertion probe. The source temperature was maintained at 250°C and the source pressure was

within the range $1.33\text{--}1.33 \times 10^{-5} \text{ N} \cdot \text{m}^{-2}$ ($1\text{--}10 \times 10^{-7} \text{ mmHg}$). Electron-beam energies of either 14eV or 70eV were used for the measurements described below. The lower-beam energy was used to identify those fragments with relatively lower appearance potentials. An accelerating potential of 8kV was used throughout this work.

Gas-liquid chromatography

A Pye series 104 gas-liquid chromatograph fitted with a flame-ionization detector was used. The carrier gas was argon at a flow rate of 60ml/min.

Fatty acid methyl esters were separated on (i) 10% polyethylene glycol adipate at 184°C and (ii) 3% SE 30 at 197°C.

The National Institutes of Health mixtures D and F were used as standards as well as reference to Burchfield & Storrs (1962). Long-chain bases were chromatographed as their trimethylsilyl derivatives on SE 30 at 200°C.

The long-chain bases and fatty acid methyl esters were prepared and separated as described by Gaver & Sweeley (1965). The trimethylsilyl derivatives of the long-chain bases were obtained by reacting the bases with *NO*-bis-(trimethylsilyl)acetamide. Long-chain bases were identified by the methods described by Morrison (1969) and Karlsson (1970) including mass spectrometry and by comparison with standards supplied by Mr. Y. Barenholz (Hadassah Medical School, Jerusalem).

Amino acid analysis

A Locarte amino acid analyser (London) was used with a sulphonated polystyrene column (1 cm × 50 cm) with an elution rate of 30ml/min. The column was eluted with NaOH-citric acid buffer (0.2M, pH 3.34) for 182min. The temperature was increased from 30–60°C and elution was continued with NaOH-citric acid buffer (0.2M, pH 4.29) for 160min and completed with 1.0M-buffer (pH 6.65) for 305min followed by regeneration with 0.2M-NaOH.

Oxidation of new lipid

The intact sphingolipid (100μg of P) was reacted for 16h at 30°C with shaking in 1ml of 2-methylpropan-2-ol-water (1:1, v/v) containing 10mg of potassium periodate/ml. The reaction mixture was taken up in 5ml of chloroform-methanol (2:1, v/v) and washed with water. A portion of the dried lower phase was subjected to t.l.c. in chloroform-methanol-water-aq. NH₃ (sp.gr. 0.88) (110:50:5:8, by vol.).

Determinations of bases and P

Long-chain bases were measured by the method of Lauter & Trams (1962) and P by the method of Bartlett (1959).

Results

The stability of the new lipid to mild alkaline and acid treatment (Dawson *et al.*, 1962) suggested that it might be a sphingolipid. There was no evidence from t.l.c. that the composition of the lipid changed after the period of alkali treatment used in its preparation. After acid hydrolysis by the procedure of Gaver & Sweeley (1965) measurement of the long-chain-base content and of P indicated that these were in equimolar proportions. The i.r. spectrum of the lipid was very similar to that of ceramide phosphorylethanolamine, showing the same characteristic secondary amide peaks at 1645 and 1545 cm^{-1} , the absence of a peak at 915 cm^{-1} and the presence of a more prominent, broader peak with shoulders in the region 3100–3400 cm^{-1} .

Mass spectra of the intact lipid and ceramide phosphorylethanolamine were very similar except for the presence of an ion at m/e 60 with the new lipid. The region of the spectra between m/e 25 and 100 for the new lipid, ceramide phosphorylethanolamine and ceramide phosphorylcholine (sphingomyelin: prepared by the method of Ansell & Hawthorne, 1964) is shown in Fig. 1. Accurate mass measurements showed that the ion at m/e 60 had the formula $\text{C}_2\text{H}_6\text{NO}$ (measured mass 60.0441; calculated 60.0444 corrected

for e_m). The presence of an ion at m/e 30 is indicative of a primary amino group and is seen in the spectra of both the unknown phospholipid and ceramide phosphorylethanolamine. The ion at m/e 44 is associated with the ceramide nitrogen and that at m/e 58 with the nitrogen in the choline moiety.

On mild acid hydrolysis (Gaver & Sweeley, 1965) of the new phospholipid or when this was attacked by a preparation of brain sphingomyelinase (Barnholz *et al.*, 1966), a ninhydrin-positive water-soluble phosphorus-containing compound was produced that behaved as a zwitterion on paper ionophoresis. This could be distinguished by paper chromatography from phosphorylethanolamine formed from ceramide phosphorylethanolamine by the same treatment. Thus in phenol saturated with water it had R_F 0.38 and gave a brownish-purple colour with ninhydrin, compared with R_F 0.55 and a purple colour for phosphorylethanolamine. On strong acid hydrolysis (5M-HCl, 48h, 100°C) the phosphorus-containing compound decomposed and the main products were recognized on paper ionophoretograms (Dawson *et al.*, 1962) as P_i and a ninhydrin-positive base that migrated towards the cathode with a mobility of about 82% of that of ethanolamine. Traces of a more slowly migrating ninhydrin-positive base were also usually seen.

Since the relative stability to acid hydrolysis of the phosphorus compound indicated that the phosphate was linked through an *O*-ester bond to the unknown base, it was assumed that the latter contained at least one hydroxyl group. However, when a large variety of known naturally occurring hydroxy amino bases were tested either chromatographically or ionophoretically on paper they showed a different behaviour from that of the unknown base. A mass spectrum of the free base gave the same prominent ion at m/e 60 corresponding to $\text{C}_2\text{H}_6\text{NO}$ (measured mass 60.0447) as was shown by the intact sphingophospholipid.

Study of the mass spectra of various amino alcohols (R. A. Klein, unpublished work) shows that an ion at m/e 60 is characteristic of a terminal vicinal hydroxyl amino grouping:

Such an ion has been demonstrated for the following amino alcohols: 2-aminopropane-1,3-diol (serinol); 3-aminopropane-1,2-diol; 2-aminobutane-1-ol; 2-aminopropane-1-ol (alaninol); 1-aminopropane-2-ol (iso-propanolamine). The homologous ion at m/e 74 has been observed with 2-methyl-2-aminopropane-1,3-diol. This ion at m/e 60 is observed only when

Fig. 1. Partial mass spectra between m/e 25 and 100, recorded at 14eV for ceramide phosphorylethanolamine (a), unknown sphingophospholipid (b) and sphingomyelin (c)

Abundances are shown relative to the largest peak in this region, and peaks of less than 5% relative abundance are not shown.

the vicinal hydroxyl amino group is present, and not without it. The relative abundance depends on the structural configuration around this grouping.

The mass-spectroscopic evidence therefore indicates that both the free base and the base in the intact phospholipid must contain a free vicinal hydroxyl amino grouping. This therefore suggested that the free base might be a dihydroxy amino compound with one of the hydroxy groups linked to a phosphate moiety in the intact phospholipid. The free base gave, like glycerol, an intense reaction with alkaline AgNO_3 (Trevelyan *et al.*, 1950), indicative of a vicinal glycol grouping; serinol and ethanolamine gave only very weak reactions. The base was readily oxidized by permanganate-periodate (von Rudloff, 1956), but no short-chain fatty acid with more than a single C atom could be detected by g.l.c. These results suggested a comparatively simple structure containing the following grouping:

The free base was then shown to have identical paper-chromatographic behaviour, in ten solvents, with authentic 3-aminopropane-1,2-diol prepared by synthesis (Table 1). It also migrated identically with this compound on paper ionophoresis at pH 3.6 (Dawson *et al.*, 1962). On cation-exchange chromatography (sulphonated polystyrene, 8% cross-linkage) on an amino acid analyser the unknown base and 3-aminopropane-1,2-diol ran identically, emerging just after histidine and before carnosine (Hamilton, 1963).

The new sphingolipid (R_F 0.49 in the t.l.c. system with chloroform-methanol-water- NH_3 as solvent)

was completely converted into a new phospholipid (R_F 0.63) by periodate oxidation. This product did not react with ninhydrin but was positive to the phospholipid reagent of Vaskovsky & Kostetsky (1968) and gave a positive carbonyl reaction when sprayed with 0.4% dinitrophenylhydrazine in 2M-HCl unlike the original compound. Ceramide phosphorylethanolamine had R_F 0.56 and was unchanged by the oxidation procedure.

Origin of the new phospholipid in the rumen

The unknown sphingophospholipid could not be detected in the diet of hay and oats on which the sheep was being maintained, nor is it a constituent of ruminant tissues. This suggested that it was formed by micro-organisms within the rumen. The lipid was found in the bacteria-rich fraction obtained from rumen contents by differential centrifuging and also in the rumen of sheep from which the ciliate protozoa had been eliminated from the rumen for several months by an initial treatment with dioctyl sodium sulphosuccinate (Abou Akkada *et al.*, 1968).

A search for bacteria containing the new phospholipid was made among species of rumen bacteria isolated by the roll-bottle technique (White *et al.*, 1970) for other purposes. The bacteria were grown in large-scale anaerobic cultures and recovered by centrifuging, and after extraction with solvent the lipids were examined by two-dimensional t.l.c. (Fig. 2). Some cultures contained ceramide phosphorylethanolamine, but only one showed appreciable amounts (30% of the total phospholipid P) of the unknown lipid on two-dimensional t.l.c. (Fig. 2). The ability to produce the lipid persisted through

Table 1. *Paper chromatography of the amino alcohol derived from the new sphingophospholipid and DL-3-aminopropane-1,2-diol*

The two compounds co-chromatographed in all solvents. Whatman no. 1 paper was used and chromatography was done in a descending direction at 20°C for 16h.

Solvent (parts by volume)	R_F or distance travelled
Methanol-pyridine-water (4:2:1)*	34cm in 16h
Ethyl acetate-acetic acid-water (14:3:3)*	23cm in 16h
Butan-1-ol-formic acid-water (10:1:6, upper phase)	0.15
Butan-1-ol-acetic acid-ethanol (12:3:5)	0.37
Phenol saturated with water-acetic acid-ethanol (50:5:6)	0.77
Phenol saturated with water-aq. NH_3 (sp.gr. 0.88) (200:1)	0.80
Butan-1-ol-pyridine-water (1:1:1)	0.43
Ethanol-water-aq. NH_3 (sp.gr. 0.88) (18:1:1)*	20cm in 16h
Butan-1-ol-acetone-diethylamine-water (10:10:2:5)	0.60
2-Methylpropan-2-ol-water-butan-2-one-diethylamine (10:10:5:1)	0.81
Propan-1-ol-water (4:1)	0.62

* These solvents ran off the end of the papers (approx. 45cm) in 16h.

Fig. 2. *T.l.c. of the lipids extracted from a Gram-negative bacterium isolated from the rumen of a sheep*
The t.l.c. plate of silica gel H was developed in two dimensions: (a) in chloroform-methanol-water (19:7:1, by vol.) followed by reactivation for 10 min at 100°C, then (b) in chloroform-methanol-11 M-NH₃ (110:50:13, by vol.). The plate, after being dried, was sprayed with ninhydrin solution and, after being heated for 5 min at 100°C was photographed and sprayed with reagent (Vaskovsky & Kostetsky, 1968) for the detection of P (spots outlined in discontinuous line): spot 1, phosphatidylethanolamine (50%); spot 2, ceramide phosphorylethanolamine (10%); spot 3, ceramide phosphoryl-3-aminopropane-1,2-diol (30%).

Table 2. *Long-chain bases present in the new sphingophospholipid and in ceramide phosphorylethanolamine*

The long-chain bases were examined by g.l.c. of *o*-trimethylsilyl derivatives on an OV1 column at 200°C.

	Composition (%) based on peak areas emerging	
	New sphingophospholipid	Ceramide phosphorylethanolamine
C ₁₇ (br.) and shorter sphinganine	8	13
C ₁₇ sphinganine		17
C ₁₈ (br.) sphinganine		11.5
C ₁₈ sphinganine		15.5
C ₁₉ (br.) sphinganine		43

many subcultures, although the percentage (20-40%) formed varied somewhat depending on the growth conditions. On isolation the lipid showed the same chemical and physical characteristics as that obtained from rumen contents. The bacterium concerned is a Gram-negative rod, but it has not, as yet, been further characterized.

Long-chain base and fatty acid composition

The long-chain bases present in the new sphingophospholipid were all saturated and contained predominantly a C₁₉ branch-chained sphinganine

comprising over 50% of those occurring (Table 2). The long-chain-base composition of ceramide phosphorylethanolamine was rather similar.

The fatty acid compositions of the two lipids are given in Table 3. Saturated and polar acids characterized by their behaviour on t.l.c. plates and on g.l.c. predominate. The new phospholipid is rich in a polar lipid that is probably a hydroxypalmitic acid (44%); a smaller amount of another polar fatty acid is possibly an oxopalmitic acid. Palmitic acid is the other major fatty acid. In ceramide phosphorylethanolamine palmitic acid predominates (40%), and only 23% was found as polar fatty acids.

Table 3. *Fatty acids present in the new sphingophospholipid and in ceramide phosphorylethanolamine*

The fatty acid methyl esters were chromatographed as described in the Materials and Methods section.

Fatty acid methyl ester	Composition (%) based on peak areas emerging	
	New sphingophospholipid	Ceramide phosphorylethanolamine
C _{14:0} (br.)	7	
C _{15:0} (br.)	13	
C _{16:0}	21	40
C _{18:0}	3	13
C _{18:1}	4	13
Unidentified	Traces	~10
Polar lipids, *probably:		
C ₁₆ (oxo)	8	7
C ₁₆ (hydroxy)	44	16

* These polar fatty acid methyl esters when chromatographed on silica gel H plates in hexane-diethyl ether-acetic acid (80:20:1, by vol.) ran in the position of a hydroxy or oxo fatty acid. They were eluted with chloroform-methanol (1:1, v/v) and had retention times relative to methyl palmitate on polyethylene glycol adipate at 184°C of 6.45 (large peak) and 5.4 (small peak), compared with methyl 12-hydroxystearate 15.4.

Discussion

Although it has not been possible to isolate large amounts of the new sphingophospholipid, there is little doubt that it consists of an *N*-acylsphingosyl-phosphate moiety esterified to one of the alcohol groupings of 3-aminopropane-1,2-diol, in other words that it is an analogue of the known sphingophospholipids sphingomyelin and ceramide phosphorylethanolamine. Sphingosyl is used in this formulation to denote a long-chain base of the sphingosine type (see IUPAC-IUB Commission on Biochemical Nomenclature, 1967) and clearly, like all of the sphingophospholipids isolated from natural sources, the lipid obtained contains a variety of long-chain bases and fatty acids. The long-chain bases, being saturated and predominantly a C₁₉ branched-chain sphinganine, are rather similar to those found in *Bacteroides melaninigenicus* by White *et al.* (1969).

Mass spectrometry of untreated phospholipid samples may be used to obtain considerable structural information about the various parts of the molecule (Klein, 1971*a,b*). At low electron-beam voltages those fragments containing nitrogen become particularly apparent (Klein, 1971*b*, 1972), since the appearance potential of nitrogen-containing fragments are, in general, lower than those of their oxygen-containing counterparts (Budzikiewicz *et al.*, 1967). In this instance the mass spectrometry of the unknown lipid clearly showed a prominent ion at *m/e* 60 corresponding to C₂H₆NO, which has also been shown (R. A. Klein, unpublished work) to be present in the spectra of compounds containing a vicinal

hydroxyl amino grouping. Since this ion undoubtedly arises from the esterified 3-aminopropane-1,2-diol present, part at least of the lipid consisted of a ceramide phosphate moiety esterified to the primary alcohol group of the base. It was still possible that some of it could be esterified to the secondary alcohol grouping of the base. Two isomers of glucosaminylphosphatidylglycerol in which D-glucosamine is linked either to the primary or secondary alcohol moiety of phosphatidylglycerol have recently been found in *Bacillus megaterium* (MacDougall & Phizackerley, 1969). However, the demonstration that the lipid could be completely converted by oxidation with periodate into a new phospholipid which is probably ceramide phosphorylglycolaldehyde indicates that the ceramide phosphate is only esterified to the primary alcoholic group of the base. Thus only a vicinal hydroxy amino compound would be oxidized with periodate.

The bacterial origin of the new phospholipid in the rumen contents seems certain. It is not present in the diet of the sheep and has never been detected in sheep tissues. Its presence in the rumen contents of a defaunated sheep indicates that the ciliate protozoa are not responsible. Finally the most convincing argument is that it can comprise a considerable portion of the phospholipid content of a bacterium originally obtained from rumen contents. Sphingolipids are very rare components of the lipids of micro-organisms. Ceramide phosphorylethanolamine has been reported as a constituent of the rumen bacteria (Dawson & Kemp, 1969). LaBach & White (1969) and White & Tucker (1970) have identified

three ceramide phosphate-containing lipids in strains of *Bacteroides melaninogenicus* namely ceramide phosphorylglycerol, ceramide phosphorylethanolamine and ceramide phosphorylglycerolphosphate.

Mr. Y. Barenholz made some preliminary observations on the long-chain bases present in the new phospholipid, and Mr. C. Howes carried out later analyses. Mr. G. Hazelwood was responsible for the isolation of the bacterial strain containing the new phospholipid. The very great assistance rendered by Mr. R. Self, Mr. K. P. Parsley, Mr. J. Eagles and Mr. B. Gordon of the Mass Spectrometry Unit at the Agricultural Research Council Food Research Institute, Norwich, U.K., is gratefully acknowledged. Mr. D. Grime and Mr. D. J. Lander are thanked for expert assistance.

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