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The Aminoethylphosphonate-Containing Lipids of Rumen Protozoa

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1. A method is presented for identifying and estimating the aminoethylphosphonate (ciliatine)-containing phospholipids in a complex mixture. 2. Evidence was obtained that the phospholipids of a pure culture of *Entodinium caudatum* and a mixed rumen protozoa sample contain diglyceride ciliatine, and a plasmalogen ciliatine was detected in the latter. 3. A ninhydrin-positive sphingolipid was isolated from rumen protozoa. Although chromatographically homogeneous on silica gel it contains two components, which were provisionally identified as ceramide ciliatine and ceramide phosphorylethanolamine. 4. A detailed phospholipid analysis of *E. caudatum* and rumen protozoa is presented. They contain no phosphatidylserine or cardiolipin, but an unidentified phosphoglyceride containing a zwitterionic amino acid is present.

The presence of the C–P bond in Nature was first revealed when Horiguchi & Kandatsu (1959) isolated 2-aminoethylphosphonate (ciliatine) from acid hydrolysates of a proteolipid prepared from ciliated protozoa of sheep rumen. Subsequently Kittredge, Roberts & Simonsen (1962) obtained evidence that the same compound was present in the sea anemone, *Anthopleura elegantissima*, from which a new phospholipid, ceramide aminoethylphosphonate (ceramide ciliatine), was isolated and characterized by Rouser, Kritchevsky, Heller & Lieber (1963). The same phospholipid has been isolated by Hori, Itasaka & Inoue (1966) from the shellfish *Corbicula sandai*, and it is now believed that compounds, including lipids, containing the C–P bond are by no means uncommon in marine organisms (Quin, 1965; de Koning, 1966; Kittredge, Isbell & Hughes, 1967). Ciliatine has also been found in total hydrolysates of *Tetrahymena pyriformis* (Kandatsu & Horiguchi, 1962), and Liang & Rosenberg (1966) have obtained evidence that diacylglyceryl 2-aminoethylphosphonate (diglyceride ciliatine) is present in these protozoa. It is probable that the same lipid accumulates in housefly larvae (*Musca domestica*) when these are given ciliatine (Bridges & Ricketts, 1966).

The present investigation was undertaken to determine the nature and concentration of the ciliatine-containing lipids present in the mixed population of rumen protozoa and also in pure cultures of the protozoon, *Entodinium caudatum*, obtained from the rumen. An extension to the method of identifying and determining phospholipids by a study of their partial degradation products (Dawson, Hemington & Davenport, 1962) proved extremely useful in this respect. Evidence was obtained that the protozoa contain not only ceramide ciliatine and diglyceride ciliatine but also a plasmalogen ciliatine and an ethanolamine-containing analogue of sphingomyelin, ceramide phosphorylethanolamine.

METHODS

Preparation of protozoa. Rumen fluid was withdrawn from fistulated adult sheep fed once daily on a diet of hay chaff (1000g.) and crushed oats (200g.). The fluid was transported to the laboratory in Dewar flasks pre-heated to 39°.

The fluid was strained through six layers of cotton gauze to remove large particles and pressed with a glass rod to extrude all large protozoa. The strained sample was placed in a stoppered conical separating funnel and maintained at 39° for 1½hr. After this time the larger protozoa had collected in an off-white mass at the bottom of the funnel. Most of the overlying suspension was removed by using a Pasteur pipette and suction, and the crude protozoal fraction was washed out and diluted with its own volume of deaerated buffer (prepared as described by Coleman, 1958). The protozoa were sedimented by centrifuging in an Autopoise (Griffin and George Ltd.). The supernatant and greenish layer were removed as completely as possible without disturbing the lower protozoal pellet. This pellet was washed three times by resuspending in 10vol. of buffer and centrifuging. A small sample of one such preparation was examined under low-power magnification and was estimated to be composed of the following: *Entodinium* 85%, *Isotricha* 7%, *Dasytricha* 6% and *Polyplastron* 2%.

Pure cultures of *E. caudatum* were kindly supplied by Dr G. S. Coleman and had been maintained by previously described methods (Coleman, 1962).

Extraction of lipids. The packed ciliates were extracted with 20vol. of $CHCl_3$–methanol (2:1, v/v) for 18hr. at room temperature. The insoluble residue was then extracted a further two times by boiling with 20vol. of the same solvent for 5min. The combined extracts were shaken with 0·2vol. of 0·9% NaCl solution and after settling overnight the clear upper phase and interface were removed from the lower $CHCl_3$ phase containing the phospholipids (Folch, Lees & Sloane-Stanley, 1957). The yield from an *E. caudatum* culture was 583μg. of lipid P/ml. packed-cell volume and the mixed rumen protozoa gave typically 228μg. of lipid P/ml. packed-cell volume.

Analysis of phospholipids. The phospholipids were degraded by incubating them successively with ethanolic NaOH, Hg^{2+}–trichloroacetic acid and methanolic HCl. After each treatment the water-soluble phosphorus-containing compounds produced were separated and estimated by procedures already described (Dawson, 1960; Dawson *et al.* 1962). The phospholipids containing C–P bonds were estimated as follows:

The amount of glyceryl aminoethylphosphonate present in the water-soluble phosphate ester fraction formed by incubating the phospholipids with ethanolic NaOH gave a measure of the diglyceride ciliatine present. On ionophoresis and chromatography (Dawson *et al.* 1962) this formed a mixed spot with the glycerylphosphorylethanolamine derived from phosphatidylethanolamine. A portion of the fraction was mixed with 0·25vol. of 5N-HCl and hydrolysed for 30min. at 100°. The acid-stable compound (glyceryl aminoethylphosphonate), remaining in the spot when the hydrolysate was evaporated to dryness and again separated by ionophoresis and chromatography, was used to compute the relative proportions of the two phospholipids. It was found that because of the salt present (the alkaline solution had been neutralized with ethyl formate) better separations were obtained if the ionophoresis preceded the chromatography after acid hydrolysis, whereas the converse is true for the separation before acid hydrolysis (Dawson *et al.* 1962). The phosphate esters liberated by Hg^{2+}–acid hydrolysis were examined in the same way (N-HCl hydrolysis at 100° for 30min.) to distinguish between the glyceryl aminoethylphosphonate and glycerylphosphorylethanolamine split off from ciliatine plasmalogen and ethanolamine plasmalogen respectively. Here, however, the usual single-dimensional paper chromatography in the phenol–acetic acid–ethanol–water solvent sufficed. The water-soluble phosphorus-containing substances present in the HCl–methanolysate were examined by single-dimensional paper chromatography in the same solvent before and after strong acid hydrolysis in a sealed tube (5N-HCl at 120° for 48hr. or more). The relative proportions of the acid-labile ethanolamine phosphate and acid-stable aminoethylphosphonate in the single spot (R_F0·55) gave a measure of the ceramide ethanolamine phosphate and ceramide aminoethylphosphonate respectively.

Thin-layer chromatography. Thin-layer chromatography of lipid extracts was carried out on silica-gel-H plates with a solvent of $CHCl_3$–methanol–water–acetic acid (65:25:4:1, by vol.) in an 'S'-chamber (Parker & Peterson, 1965) at 4°. After being sprayed with ninhydrin to reveal amino lipids the plates were sprayed with the reagent of Dittmer & Lester (1964) to detect phospholipids.

Preparation of sphingophospholipid (amino). An enriched sample of the ninhydrin-positive sphingophospholipids was obtained by chromatography on silicic acid by the method of Hanahan, Dittmer & Warashina (1957). It was eluted with 40% (v/v) methanol in $CHCl_3$. The sample was treated chemically to remove contaminating phosphatidylethanolamine and ethanolamine plasmalogen. Approx. 3mg. of phospholipid P was dried down and incubated for 1hr. at 40° with CCl_4 (1·6ml.), ethanol (16ml.), N-NaOH (0·8ml.) and water (1·3ml.). After incubation ethyl formate (1ml.), $CHCl_3$ (32ml.) and water (10ml.) were added and the mixture was shaken. The lower layer, containing the alkali-stable phospholipids, was then shaken for 30min. at 37° with an equal volume of methanol–50mM-$HgCl_2$ (in 0·1N-HCl)–$CHCl_3$ (45:48:3, by vol.). The lower phase was separated and washed once with an equal volume of the same solvent mixture in which 0·9% NaCl was substituted for the $HgCl_2$ in HCl. The lower phase contained neutral lipids, fatty acids and the sphingophospholipids.

A column of silicic acid (4g.) was poured in $CHCl_3$–methanol (2:1, v/v) and washed with $CHCl_3$. The sample of acid- and alkali-treated lipid containing about 1mg. of phospholipid P was applied in $CHCl_3$ and the column eluted successively with 20ml. portions of $CHCl_3$, 20% (v/v) methanol in $CHCl_3$, 40% (v/v) methanol in $CHCl_3$ and methanol. The ninhydrin-positive sphingophospholipid was eluted by 40% methanol in $CHCl_3$.

RESULTS

Thin-layer chromatography of lipid extracts. Chromatography of the rumen protozoal lipids on silica gel H suggested a comparatively simple phospholipid composition. There were two main spots of lecithin (R_F0·23) and ethanolamine phosphoglyceride (R_F0·86), in between which ran a small spot of ninhydrin-positive phospholipid (R_F0·49).

Chemical degradation of lipid extracts. The hydrophobic residues were removed from the phospholipids present in the mixture by various chemical treatments and the water-soluble phosphorus-containing components examined by paper chromatography and ionophoresis (Dawson *et al.* 1962).

(*a*) Alkaline ethanolysis. When the lipid extracts from pure cultures of *E. caudatum* and mixed rumen protozoa were treated with ethanolic sodium hydroxide, examination of the water-soluble products formed from the 'phosphatidyl' compounds by paper chromatography and ionophoresis showed the presence of large spots of glycerylphosphorylcholine (derived from lecithin) and glycerylphosphorylethanolamine. However, when the water-soluble phosphate esters were subjected to acid hydrolysis (N-hydrochloric acid at 100° for 20min.) part of the 'glycerylphosphorylethanolamine' spot remained stable and was not decomposed even on prolonging the hydrolysis for 2hr. On the other hand all of the glycerylphosphorylcholine spot disappeared after the 20min. hydrolysis. Glycerylphosphorylethanolamine is known

to be completely hydrolysed into glycerophosphoric acid and ethanolamine after hydrolysis in N-hydrochloric acid at 100° for 10min. (Schmidt, Bessman & Thannhauser, 1953), so it seems reasonable to assume that the ninhydrin-positive phosphate ester stable to acid represents the glyceryl ester of aminoethylphosphonate. Because of the close similarity of the structure of this substance to glycerylphosphorylethanolamine it is to be expected that it would move to almost identical positions on chromatography and ionophoresis (Kittredge *et al.* 1962). On the other hand the C–P bond in the aminoethylphosphonate ester would be quite resistant to acid hydrolysis compared with the C–O–P bond in the corresponding glyceryl ester of ethanolamine phosphate (Kittredge *et al.* 1962; Quin, 1965). Control experiments showed that, in the corresponding chromatograms prepared from guinea-pig brain and sheep liver phospholipids, the glycerylphosphorylethanolamine spot completely disappeared on hydrolysis of the solution in N-hydrochloric acid at 100° for 20min.

The deacylation products of the diacylphosphoglycerides also indicated that the protozoa contain small amounts of phosphatidic acid and phosphatidylinositol. No glycerylphosphorylserine was produced, indicating the absence of phosphatidylserine. However, there was present among the products a ninhydrin-positive phosphorus-containing compound that we have not observed previously in any similar degraded phospholipid sample [R_F 0·68 in phenol–acetic acid–ethanol–water solvent (Dawson *et al.* 1962); M_{P_i} 0·58 on ionophoresis at pH 3·6]. On acid hydrolysis (N-hydrochloric acid at 100° for 20min.) the compound decomposed completely and the products appeared on ionophoresis to be glycerophosphate and a zwitterionic (pH 3·6) amino acid. Up to the present it has not been possible to identify this amino acid by ion-exchange chromatography on an amino acid analyser (Hamilton, 1963) or by paper chromatography (Smith, 1960). The protozoal phospholipid fractions contain only a few per cent of the unidentified lipid from which it is derived, and further work on the isolation and identification of the parent phospholipid is proceeding.

(*b*) Hg^{2+}–trichloroacetic acid hydrolysis. The lysoplasmalogens remaining after alkaline ethanolysis of the rumen protozoal lipids were hydrolysed into long-chain aldehydes and water-soluble phosphate esters with a Hg^{2+}–trichloroacetic acid reagent (Dawson *et al.* 1962). Examination of the phosphate esters by chromatography and ionophoresis revealed one ninhydrin-positive spot in the position of glycerylphosphorylethanolamine. However, on acid hydrolysis of the product (N-hydrochloric acid at 100° for 30min.), 14·9% remained stable, whereas the remainder showed the expected behaviour of glycerylphosphorylethanolamine and decomposed into ethanolamine and glycerophosphoric acid. On prolongation of the hydrolysis to 2hr., 14·6% of the compound remained stable. This suggests therefore that the hydrolysate contains both glycerylphosphorylethanolamine and the acid-resistant glyceryl ester of aminoethylphosphonate derived from a ciliatine plasmalogen. The lipid extract of *E. caudatum* contained very little plasmalogen phospholipids and it was not possible to ascertain whether any of the aminoethylphosphonate-containing plasmalogen was present.

(*c*) Acid methanolysis. On examination of the water-soluble phosphorus-containing substances split off from the residual rumen protozoal phospholipids by methanolic hydrochloric acid (Dawson *et al.* 1962) the main product was a ninhydrin-positive compound that was chromatographically indistinguishable from phosphorylethanolamine. Since this fraction is usually formed from sphingophospholipids, this result suggested that an ethanolamine-containing analogue of sphingomyelin was present in the lipid extract. A sample of the 'phosphorylethanolamine-like' compound was isolated from the hydrolysate by band ionophoresis followed by elution (Dawson & Dittmer, 1961). Its stability to a long hydrolysis with 5N-hydrochloric acid at 120° was then tested. Under such hydrolytic conditions phosphorylethanolamine is slowly hydrolysed (de Koning & McMullan, 1965), whereas aminoethylphosphonate is resistant (Kittredge *et al.* 1962). On ionophoresis of the hydrolysis products, only part of the isolated substance was hydrolysed by 5N-hydrochloric acid

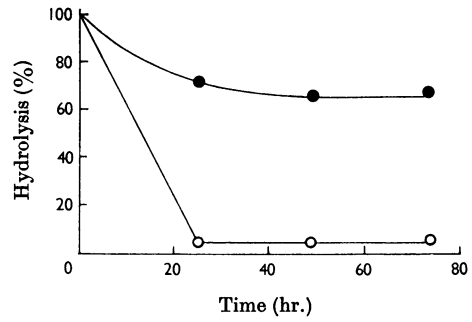

Fig. 1. Acid hydrolysis of synthetic phosphorylethanolamine (○) and the 'phosphorylethanolamine-like' substance (●) isolated from rumen protozoal sphingolipids. Samples were sealed into glass tubes with 1ml. of 5N-HCl and heated at 120°. The acid was removed and the residue subjected to paper ionophoresis at pH 3·6. Inorganic phosphate and any unhydrolysed phosphorus spots were localized and their P content estimated (Dawson *et al.* 1962).

with the liberation of inorganic phosphate and ethanolamine; the remainder was completely stable to the acid (73½hr.) (Fig. 1). Under the same conditions of hydrolysis synthetic phosphorylethanolamine was completely decomposed within 24hr. These results suggest that the protozoa possess both phosphorylethanolamine-containing and aminoethylphosphonate-containing sphingolipids.

Properties of the isolated sphingophospholipid. The alkali-stable sphingophospholipid prepared from rumen protozoa ran as a single spot on thin-layer chromatography with the ninhydrin-positive and phosphorus-positive areas coinciding. Its infrared spectrum showed typical amide peaks at 1545 and 1645cm.$^{-1}$. It was identical with that of the published spectrum of ceramide ciliatine isolated from shellfish (Hori *et al.* 1966) except that it had an additional or more prominent small peak at 1032cm.$^{-1}$. Since ceramide phosphorylethanolamine can be expected to give a similar infrared spectrum, further evidence was required. On methanolysis with methanolic hydrochloric acid the sole phosphorus-containing product showed chromatographic properties identical with those of synthetic phosphorylethanolamine. However, in confirmation of the previous result obtained with the 'phosphorylethanolamine-like' substance obtained from the phospholipids stable to mild alkaline and Hg^{2+}–acid treatment, strong acid hydrolysis indicated that it was a mixture of acid-labile and acid-stable forms. Under hydrolytic conditions that completely decomposed phosphorylethanolamine (5N-hydrochloric acid at 120° for 42hr.) only 35% decomposed and the residual substance remained stable after 76hr. hydrolysis. The decomposition products of the hydrolysable portion were inorganic phosphate and a ninhydrin-positive base that was identified as ethanolamine by its electrophoretic mobility and its co-chromatography (R_F 0·37) with ethanolamine on paper in butanol–acetic acid–water (12:3:5, by vol.).

Phospholipid composition of E. caudatum *and mixed rumen protozoa.* The phospholipid analyses of the protozoal lipid fractions are presented in Table 1. The sample of *E. caudatum* contained minimal bacterial contamination and is likely to be much purer with respect to protozoal lipid than the mixed rumen protozoal fraction, which was grossly contaminated with bacteria and food particles.

Lecithin is the predominant individual lipid present in both extracts although it is surpassed by the ethanolamine phosphate-containing and aminoethylphosphonate-containing phosphoglycerides considered as a single class. Both samples contain no phosphatidylserine and cardiolipin; the absence of the latter is probably due to the fact that the organisms are strict anaerobes and contain no mitochondria. The unidentified phosphoglyceride, which contains an unknown neutral amino acid as part of its structure, was present at a higher percentage concentration in the *E. caudatum* sample. Because the latter sample contained a greater protozoal content this could indicate that the unidentified phospholipid is a true protozoal component.

Table 1. *Individual phospholipids present in protozoal lipid extracts*

	E. caudatum (% of lipid P)	Mixed rumen protozoa (% of lipid P)
Phosphoglycerides		
Phosphatidylcholine	26·1	36·3
Phosphatidylserine	0	0
Phosphatidylinositol	3·9	3·1
Phosphatidic acid	3·0	1·4
Phosphatidylethanolamine	19·7	18·7
Diglyceride aminoethylphosphonate	18·0	11·0
Ethanolamine plasmalogen	1·2	9·5
Aminoethylphosphonate plasmalogen		1·9
Choline plasmalogen	1·0	0·9
Glyceryl ether phospholipids	5·4	4·5
Unidentified; containing neutral amino acid	5·3	3·6
Sphingophospholipids		
Ceramide aminoethylphosphonate	6·4	4·1
Ceramide ethanolamine phosphate	4·3	2·2
Sphingomyelin	5·3	2·0
Unidentified	0·8	0·8

DISCUSSION

The complex pattern of phospholipids present in protozoal lipid extracts revealed by chemical degradation emphasizes the oversimplified picture that can be presented when lipid extracts in general are only examined by thin-layer chromatography. This especially applies in the present analyses where the ethanolamine phosphate-containing and aminoethylphosphonate-containing glycerides possess almost identical chromatographic properties on silicic acid yet can be resolved into five individual phospholipids.

The present results clearly confirm that the rumen protozoa are rich in phospholipids containing the aminoethylphosphonate or ciliatine moiety (Horiguchi & Kandatsu, 1959). The properties of the ciliatine-containing phosphoglycerides present indicate that the ciliatine can, in an analogous manner to phosphorylethanolamine, be esterified to a diglyceride or an acylalkenylglycerol moiety. The ultimate proof of this must, however, depend on the isolation of the pure compounds and their detailed chemical characterization.

Diglyceride ciliatine and phosphatidylethanolamine occur in the molar ratios 1:1·7 in rumen protozoa and 1:1·1 in *E. caudatum*. This is considerably more diglyceride ciliatine than in the only other reported source, *Tetrahymena pyriformis*, where the corresponding ratio is only 1:13 (Liang & Rosenberg, 1966). The ciliatine plasmalogen represents only about 15% of the amino-plasmalogen fraction of rumen protozoa (ciliatine plasmalogen + ethanolamine plasmalogen). It has hitherto not been described as a naturally occurring phospholipid.

The evidence presented suggests that the sphingophospholipid fraction isolated contains two compounds with free amino groups. One of these, ceramide aminoethylphosphonate, contains the acid-resistant C–P bond; it has been described previously only as a constituent of marine organisms (Rouser *et al.* 1963; Hori *et al.* 1966). The other, constituting 35–40% of the P of the total fraction, is probably ceramide phosphorylethanolamine, which contains the acid-labile C–O–P ester bond. The presence of this bond probably accounts for the extra peak (or greater prominence) of the band at 1032cm.$^{-1}$ in the infrared spectrum of the substances isolated compared with the spectrum of the ceramide aminoethylphosphonate obtained from shellfish. This band has been ascribed to the P–O–C (alkyl) bond (Bellamy, 1958). Baer & Stanacev (1964) compared the infrared spectra of synthetic diglyceride ciliatine and phosphatidylethanolamine and also found the only difference was a small peak at 1030cm.$^{-1}$ in the latter. Ceramide phosphorylethanolamine has not previously been found in Nature, although Crone & Bridges (1963) obtained some evidence suggesting that a sphingolipid containing phosphorus and ethanolamine occurs in the housefly, *Musca domestica*. We have obtained evidence from hydrolytic studies that a similar compound also occurs in the blowfly, *Calliphora erythrocephala*.

The observation that protozoa contain both ciliatine-containing lipids and their phosphorylethanolamine-containing analogues could suggest that they can synthesize both ciliatine and phosphorylethanolamine. Because of the very similar structures of these two compounds it is possible that the enzyme synthesizing CDP-ethanolamine would not discriminate between them and thus CMP-ciliatine would be synthesized as well (Liang & Rosenberg, 1966; Bridges & Ricketts, 1966). Both compounds could then react with lipid acceptors (diglyceride, acylalkenylglycerol, ceramide), forming the phospholipids found. Against this hypothesis, however, must be considered the preliminary results of Rosenberg (1964), who found that the lipid ciliatine of *Tetrahymena pyriformis* turned over ^{32}P faster than did the water-soluble ciliatine, which is hardly consistent with a precursor role of the latter. By analogy, it is possible that ciliatine-containing phospholipids are also present in the small glyceryl ether fraction of protozoa.

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