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Isolation of a New Phospholipid, Phosphatidyl-*N*-(2-hydroxyethyl)-alanine, from Rumen Protozoa

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1. Phosphatidyl-*N*-(2-hydroxyethyl)alanine was isolated from the mixed protozoal fraction of rumen and characterized. 2. Of the fatty acids 31% was octadecenoic acid, 91% of which was the *trans*-Δ^{11}-isomer, an intermediary in the ruminal hydrogenation of linolenic acid and linoleic acid.

During an investigation of the ciliatine-containing phospholipids of *Entodinium caudatum* and mixed rumen protozoa a phosphorus-containing spot was observed on paper chromatography and ionophoresis of the deacylation products that did not correspond to those produced from any known phospholipid (Dawson & Kemp, 1967). The parent phospholipid has now been isolated from a mixed rumen protozoal fraction and its structure established as phosphatidyl-*N*-(2-hydroxyethyl)alanine.

EXPERIMENTAL

Preparation of rumen protozoal fraction and extraction of lipids. Rumen contents of cattle, and on one occasion of sheep, were collected from a local slaughterhouse and up to 80l. was brought to the laboratory in a plastic dustbin. Rumen contents of slaughtered animals showed considerable variations in their complement of viable protozoa, and consequently samples were selected after low-power microscopic examination immediately after the rumen had been opened. The contents were centrifuged in a large basket centrifuge (mesh 2mm.) to separate the rumen liquor from large unfermented food particles. This liquor was centrifuged at 1500rev./min. for 2min. in a MSE Mistral 6L centrifuge (head 59563) and the off-white pellet collected. The crude protozoa were washed by suspending in a buffer solution (Coleman, 1958), the volume of which was one-tenth of the original rumen liquor. The washing was repeated.

The lipids were extracted from the protozoal pellet by two methods. (1) The pellet was extracted twice with 20vol. of chloroform–methanol (2:1, v/v) for 30min. at room temperature. (2) The pellet was extracted twice with 5vol. of cold acetone, the extracts were discarded and the residue was extracted three times with 5vol. of chloroform–methanol (2:1, v/v) for 20min. at room temperature. The latter method was more economical in solvent and eliminated some of the neutral lipids, but the possibility exists of artifact phospholipid formation (Helmy & Hack, 1966; Wendt & Debuch, 1968).

Isolation of the new phospholipid. The chloroform–methanol extract of the protozoa was evaporated to dryness and the residue extracted with chloroform–methanol (2:1, v/v) (800ml. for 2·8l. of original protozoal fraction). The mixture was shaken with 200ml. of 0·1M-$CaCl_2$ and the lower phase separated. The upper phase was washed with an equal volume of theoretical lower phase, and the two lower phases containing the calcium salts of the phospholipids were evaporated to dryness (0·82g. of lipid P). The lipids were applied to a silicic acid (Mallinckrodt) column (800g.; 45cm. long × 5·5cm. diam.) in chloroform, and this was successively eluted with: (1) 1·6l. of chloroform; (2) 1·6l. of chloroform–methanol (19:1, v/v); (3) 1·6l. of chloroform–methanol (9:1, v/v); (4) 1·6l. of chloroform–methanol (1:4, v/v). The progress of the separation was monitored by paper ionophoresis of the deacylation products (Dawson, Hemington & Davenport, 1962) and t.l.c. on silica gel in chloroform–methanol–water (19:7:1, by vol.). The new phospholipid appeared in fraction 3 along with some phosphatidylinositol, phosphatidylethanolamine and pigmented material (47mg. of P). The lipids recovered from this fraction were dissolved in 100ml. of chloroform–methanol (2:1, v/v) and washed with 25ml. of 0·1M-EDTA (sodium salt), pH7·5. The lower phase was removed and combined with the lower phase from a rewashing of the upper phase with an equal volume of the theoretical lower phase. The lipids (sodium salts) were recovered and applied in chloroform to a silicic acid (Mallinckrodt) column (100g.; 30cm. long × 2·5cm. diam.). The column was eluted successively with: (1) 100ml. of chloroform; (2) 300ml. of chloroform–methanol (4:1, v/v); (3) 500ml. of chloroform–methanol (3:2, v/v). The first elution removed some colour, the second removed colour and phosphatidylethanolamine and the third eluted the new phospholipid, followed later by phosphatidylinositol. The fractions containing the new phospholipid were combined and evaporated to dryness, yielding 27mg. of lipid P, representing a yield of 3·4% of the original protozoal phospholipid. Direct analysis of the lipids of mixed rumen protozoa from a sheep had shown that the new phospholipid constituted 3·6% of the total lipid P (Dawson & Kemp, 1967).

Preparation of deacylated fragment from new phospholipid. The phospholipid (0·2mg. of P) was subjected to alkaline

methanolysis, the water-soluble fragment was separated and the alkali removed on an Amberlite IRC 50 column as described by Dawson *et al.* (1962). The eluate was concentrated to small volume and the deacylated fragment separated by running it as a 10 cm. streak on a preparative paper chromatogram [phenol saturated with water–acetic acid–ethanol (50:5:6, by vol.), descending overnight]. The deacylated product (R_F 0·72) was located with quinine hydrochloride (Rorem, 1959) and eluted from the paper with water.

Preparation of nitrogen-containing moiety from new phospholipid. The phospholipid (10 mg. of P) was dissolved in 5 ml. of chloroform and 3 ml. of methanol, and 2 ml. of methanolic 0·5 M-NaOH added. After the mixture had stood at room temperature for 1 hr. to complete the deacylation, 7 ml. of chloroform, 13 ml. of ethanol and 15 ml. of water were added. After shaking the mixture was centrifuged, and the upper layer was collected and passed down a column (10 cm. long × 1 cm. diam.) of Amberlite IRC-50 (NH_4^+ form) to remove Na^+. The deacylated fragment was washed through the column with 25 ml. of methanol–water (1:1, v/v). The eluate was evaporated to dryness *in vacuo* and to the residue was added 5 ml. of M-HCl. The mixture was hydrolysed for 2 hr. at 100° and the acid was then removed *in vacuo* over KOH.

The residue was dissolved in 25 ml. of water adjusted to pH 2 with M-HCl and passed down a recently regenerated and washed column (25 cm. long × 1 cm. diam.) of Zeo-Karb 225 (H^+ form). After the column had been washed with water, the N-containing component was eluted with aq. 5 M-NH_3. The eluate was evaporated to dryness and the product was crystallized from hot methanol. About 7 mg. of thin colourless needles was obtained.

Synthesis of DL-N-(2-*hydroxyethyl*)*alanine.* The synthesis was similar to the method described by Vièles & Séguin (1954). To 5 mM-2-chloropropionic acid and 10 mM-$NaHCO_3$ in 5 ml. of water was added 5 mM-ethanolamine, and the mixture was heated for 18 hr. at 80°. After dilution to 200 ml., the *N*-(2-hydroxyethyl)alanine was adsorbed on a column (25 cm. long × 2·5 cm. diam.) of Dowex 1 (OH^- form) and the column was washed with 500 ml. of water. It was stripped from the column along with unchanged chloropropionic acid with 0·5 M-HCl. The solution was evaporated to dryness and, after the residue had been dissolved in 100 ml. of water, the solution was adjusted to pH 2 and applied to a column (25 cm. long × 1 cm. diam.) of Zeo-Karb 225 (H^+ form). The column was washed with water to complete the removal of the chloropropionic acid, and the *N*-(2-hydroxyethyl)alanine was then eluted with aq. 5 M-NH_3 (250 ml.). The eluate was evaporated to dryness, and the residue was treated with 5 ml. of ice-cold methanol to remove colour. The residue was dissolved in hot methanol and the volume reduced until crystallization just began (3 ml.). The DL-*N*-(2-hydroxyethyl)alanine crystallized at 0° as colourless hexagons, m.p. 193° (uncorr.); Vièles & Séguin (1954) report m.p. 193–195° and Maekawa & Tsumura (1956) m.p. 193° for the DL-compound.

In a second preparation the *N*-(2-hydroxyethyl)alanine was isolated from the crude reaction mixture by preparative paper ionophoresis. Portions (2·5% of the reaction mixture) were applied as 30 cm. bands on Whatman no. 1 papers, and subjected to electrophoresis in a pyridine–acetic acid buffer, pH 3·6. The origin strips were cut out and eluted chromatographically with water. The water eluate was evaporated to dryness and the residual *N*-(2-hydroxyethyl)alanine recrystallized from hot methanol.

Analysis. Phosphorus was determined by the method of Bartlett (1959) after oxidation of the compounds by $HClO_4$. Fatty acids were determined by titration after the phospholipids had been hydrolysed and the released acids extracted into light petroleum (Dittmer & Dawson, 1961). T.l.c. was carried out in a saturation chamber (Parker & Peterson, 1965); I_2 vapour was used for the non-specific location of all lipids. Phospholipids on the thin layer were detected by the spray described by Vaskovsky & Kostetsky (1968). Paper ionophoresis was carried out for 1 hr. under white spirit at 40 v/cm. (Dawson *et al.* 1962).

RESULTS

The new phospholipid appeared to be chromatographically homogeneous when examined by t.l.c. on silica gel H in several solvents. However, it could not be identified with any known phospholipid, and when the water-soluble fragment from alkaline ethanolysis was subjected to paper chromatography and ionophoresis (Dawson *et al.* 1962) its behaviour did not correspond to the equivalent product obtained from any known phospholipid. On paper ionophoresis the deacylated product had a net negative charge at pH 3·6 with a mobility relative to inorganic phosphorus of 0·59. It gave a ninhydrin reaction on paper, although the intact lipid gave no discernible reaction in comparison with phosphatidylethanolamine and phosphatidylserine on silica gel plates and only a faint reaction on formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959).

On acid hydrolysis (1 M-hydrochloric acid for 20 min.) the deacylated fragment broke down completely, giving two products that could be readily separated by paper ionophoresis at pH 3·6. The first was an electrically neutral ninhydrin-reacting compound devoid of phosphorus, and the second contained phosphorus and migrated to the same position as α-glycerophosphate (M_{P_i} 0·81). On paper chromatography in a phenol saturated with water solvent the phosphorus-containing compound again ran with α-glycerophosphate (R_F 0·35) whereas the ninhydrin-reacting substance ran near the solvent front (R_F 0·89).

Studies with unimolecular films of the intact phospholipid on a Langmuir trough indicated that it had an area per molecule, at collapse pressure (45 dynes/cm.), of 49 Å^2. This is the area typically occupied by diacyl-phosphoglycerides (Shah & Schulman, 1965). The attraction of $^{45}Ca^{2+}$ to the monolayer suggested that each hydrophilic head group of the phospholipid molecule had one net negative charge (Hauser & Dawson, 1967).

Titration of the fatty acids liberated after acid hydrolysis, with methyl palmitate as the standard, yielded a fatty acid/phosphorus ratio of 2·05 μmoles/

μg.atom of phosphorus, which again indicated a diacyl-phosphoglyceride structure. Acetolysis of the phospholipid (Renkonen, 1965) yielded a main lipid product that migrated to the same position as diglyceride acetate on t.l.c. on silica gel G with light petroleum (b.p. 40–60°)–ether (4:1, v/v) as solvent. No monoglyceride diacetate was observed. The fatty acids liberated on total saponification were analysed by g.l.c. and consisted mainly of palmitic acid (40%), stearic acid (14%), octadecenoic acid (31%) and linoleic acid (3·5%) with at least 13 other acids in very minor amounts. The octadecenoic acids were examined (Kemp & Dawson, 1968) and consisted mainly of *trans*-isomers (91%), the *trans*-Δ^{11}-isomer being practically the only component of this fraction. The *cis*-isomers (9%) contained 72% Δ^{9}-octadecenoic acid (oleic acid) and 28% Δ^{11}-octadecenoic acid.

Identification of nitrogenous component. The nitrogenous component isolated from the new phospholipids appeared to be chromatographically homogeneous on paper, and it remained stable on strong acid hydrolysis (5M-hydrochloric acid for 18hr. at 110° or for 40hr. at 120°). It could not be equated with any naturally occurring known ninhydrin-reacting substance either by paper chromatography (Smith, 1960) or by cation-exchange chromatography (Hamilton, 1963). On paper ionophoresis the nitrogenous component was electrically neutral at pH 3·6 but had a net positive charge at pH 1·5 and a net negative charge at pH 9·6, behaviour typical of a monoamino monocarboxylic amino acid. On paper, the ninhydrin colour was weak compared with the usual colour yield of an amino acid. Moreover, on testing with the acetaldehyde–nitroprusside spray for secondary amines (Macek, Hacaperkova & Kakáč, 1956) a distinct positive reaction was obtained. The nitrogenous component was not oxidized by ammoniacal sodium periodate under conditions in which serine was readily decomposed. It did not form a complex with copper carbonate and was therefore presumably not a simple α-amino acid (Crumpler & Dent, 1949). Tests for thiol or disulphide groupings (Toennies & Kolb, 1951) were negative.

The n.m.r. spectra of the unknown in D_2O showed a relatively simple spectrum with eight non-exchangeable protons, and was interpreted to mean that the compound contained a $CH_3 \cdot CH \cdot$ grouping separated from $\cdot CH_2 \cdot CH_2 \cdot$ by a hetero atom or non-protonated carbon atom. Since the unknown presumably contained a secondary amine group and a hydroxyl group for esterification with the glycerophosphate in the phospholipid structure, this suggested that the compound might be *N*-(2-hydroxyethyl)alanine. Elementary analyses agreed well (Found: C, 45·0; H, 8·4; N, 10·3; O, 36·3. Required: C, 45·1; H, 8·3; N, 10·5; O, 36·1%). The identification was confirmed by the following observations involving the unknown nitrogenous component and synthetic DL-*N*-(2-hydroxyethyl)alanine: (1) they were inseparable on paper chromatography in 11 different solvent systems (Table 1); (2) they both ran to the same position on ion-exchange chromatography on sulphonated polystyrene (8% cross-linkage); they

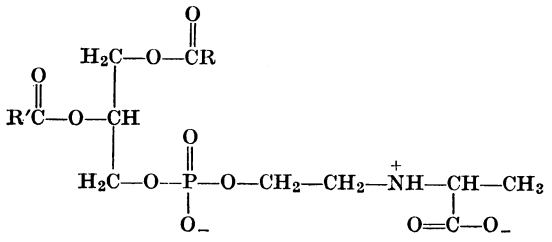
Table 1. *Paper chromatography of the nitrogen-containing moiety from the new phospholipid, DL-N-(2-hydroxyethyl)alanine and L-alanine*

Solvent (parts, by vol.)	R_F		
	DL-*N*-(2-Hydroxyethyl)-alanine	Nitrogenous component isolated from new phospholipid	L-Alanine
Phenol saturated with water–acetic acid–ethanol (50:5:6)	0·86	0·86	0·65
Butan-1-ol–pyridine–water (1:1:1)	0·46	0·47	0·29
Butan-1-ol–formic acid–water (10:1:6, upper phase)	0·25	0·25	0·265
Methanol–water–pyridine (4:1:2)	0·66	0·66	0·51
Ethanol–water–aq. NH_3 (sp.gr. 0·88) (18:1:1)	0·44	0·44	0·28
Phenol saturated with water–aq. NH_3 (sp.gr. 0·88) (200:1)	0·78	0·78	0·62
2-Methylpropan-2-ol–water–butan-2-one-diethylamine (10:10:5:1)	0·72	0·72	0·64
Butan-1-ol–acetic acid–water (12:3:5)	0·34	0·34	0·31
Ethyl acetate–acetic acid–water (14:3:3)	0·49	0·49	0·44
Butan-1-ol–acetone–diethylamine–water (10:10:2:5)	0·41	0·40	0·34
Propan-1-ol–water (4:1)	0·41	0·41	0·34

were eluted from the column near to the ethanolamine peak and just before ammonia; (3) they gave identical n.m.r. spectra in D_2O.

DISCUSSION

The demonstration that from the new phospholipid the various degradation procedures can produce fatty acids (2 moles/μg. atom of phosphorus), diglyceride, glycerophosphate and the glycerylphosphoryl ester of *N*-(2-hydroxyethyl)-alanine suggests that it is a phosphoglyceride containing a phosphatidyl or diacyl-glycerophosphate moiety. The properties of the deacylated phosphorus-containing fragment suggest that this is linked to the *N*-(2-hydroxyethyl)alanine through an *O*-ester bond. By analogy with other phosphoglycerides found in Nature the new phospholipid therefore probably has structure (I). This possesses



(I)

a net negative charge on its hydrophilic region, and since phosphatidylserine is absent from rumen protozoa (Dawson & Kemp, 1967) it might substitute for this phospholipid in the membranes of these micro-organisms. The phospholipid may be regarded as a *N*-substituted phosphatidylethanolamine and could be formed metabolically from this phosphoglyceride. Helmy & Hack (1966) and Wendt & Debuch (1968) have described an *N*-substituted phosphatidylethanolamine formed as an artifact by the reaction of mesityl oxide present in the acetone used to dehydrate the tissue before lipid extraction. However, the phospholipid described here has a different structure and is present in extracts where acetone has not been used to dehydrate the protozoa. So far it has not been found in any source apart from rumen protozoa. It was not present in cultures of the aerobic protozoan *Tetrahymena pyriformis*.

The finding that the *trans*-Δ^{11}-octadecenoic acid accounts for nearly 90% of the total octadecenoic acid fraction found in the phospholipid is of note because this acid is an intermediary in the hydrogenation of dietary linolenic acid and linoleic acid to stearic acid in the rumen (Kemp & Dawson, 1967). Although whole rumen digesta are rich in this octadecenoic acid isomer, the total phospholipid fraction from rumen bacteria contains only some 14% of the *trans*-Δ^{11}-compound (Katz & Keeney, 1966).

As far as we are aware *N*-(2-hydroxyethyl)alanine has not previously been described as occurring in Nature.

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REFERENCES

Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
Coleman, G. S. (1958). *Nature, Lond.*, **182**, 1104.
Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.
Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
Dawson, R. M. C. & Kemp, P. (1967). *Biochem. J.* **105**, 837.
Dittmer, J. C. & Dawson, R. M. C. (1961). *Biochem. J.* **81**, 535.
Hamilton, P. B. (1963). *Analyt. Chem.* **35**, 2055.
Hauser, H. & Dawson, R. M. C. (1967). *Europ. J. Biochem.* **1**, 61.
Helmy, F. M. & Hack, M. H. (1966). *Lipids*, **1**, 279.
Hörhammer, L., Wagner, H. & Richter, G. (1959). *Biochem. Z.* **331**, 155.
Katz, I. & Keeney, M. (1966). *J. Dairy Sci.* **49**, 962.
Kemp, P. & Dawson, R. M. C. (1967). *Abstr. 11th Int. Congr. Biochem. Problems of Lipids, Jerusalem.*
Kemp, P. & Dawson, R. M. C. (1968). *Biochem. J.* **109**, 477.
Macek, K., Hacaperkova, J. & Kakáč, B. (1956). *Pharmazie*, **11**, 533.
Maekawa, K. & Tsumura, S. (1956). *Bull. agric. Chem. Soc., Japan*, **20**, 101.
Parker, F. & Peterson, N. F. (1965). *J. Lipid Res.* **6**, 455.
Renkonen, O. (1965). *J. Amer. Oil Chem. Soc.* **42**, 298.
Rorem, E. S. (1959). *Nature, Lond.*, **183**, 1739.
Shah, D. O. & Schulman, J. H. (1965). *J. Lipid Res.* **6**, 341.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 83. London: William Heinemann (Medical Books) Ltd.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
Vaskovsky, V. E. & Kostetsky, E. Y. (1968). *J. Lipid Res.* **9**, 396.
Vièles, P. & Séguin, J. (1954). *C.R. Acad. Sci., Paris*, **238**, 1819.
Wendt, V. G. & Debuch, H. (1968). *Hoppe-Seyl. Z.* **349**, 789.