yet decided whether in fact the isoprenoid alcohol can be formed from mevalonic acid in the chloroplasts and also whether this alcohol (possibly in the form of a pyrophosphate) can then condense with a quinone to form plastoquinone. In animal tissues the corresponding condensation to form ubiquinone occurs in the mitochondria (Stoffel & Martius, 1960).

SUMMARY

1. Three fractions were isolated from fresh leaves of tobacco: (a) one containing chloroplasts, (b) one containing mitochondria and broken chloroplasts, and (c) a supernatant fraction.

2. Both plastoquinone (424 μ moles/kg. of leaf tissue) and solanesol (86 μ moles/kg. of leaf tissue) were concentrated in the chloroplasts.

3. Both compounds were the homologues containing nine isoprenoid units, i.e. solanesol-45 ($C_{45}H_{73}OH$) and plastoquinone-45 each with a C_{45} side chain.

4. All the isolated solanesol was unesterified.

5. Some plastoquinone (34 μ moles/kg. of fresh leaves) and solanesol (15 μ moles/kg. of fresh leaves) found in the supernatant fraction were not associated with the chloroplasts.

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REFERENCES

- Aiyar, A. S. & Sreenivasan, A. (1961). *Nature, Lond.*, **190**, 344.
- Aiyar, A. S. & Sreenivasan, A. (1962a). *Biochem. J.* **82**, 179.
- Aiyar, A. S. & Sreenivasan, A. (1962b). *Biochem. J.* **82**, 183.
- Arnon, D. I. (1961). *Fed. Proc.* **20**, 1012.
- Bishop, N. I. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 501.
- Bishop, N. I. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1696.
- Bishop, N. I. (1961). *Ciba Found. Symp.: Quinones in Electron Transport*, p. 385.
- Clark, V. M. & Todd, A. (1961). *Ciba Found. Symp.: Quinones in Electron Transport*, p. 130.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Crane, F. L. (1959a). *Plant Physiol.* **34**, 546.
- Crane, F. L. (1959b). *Plant Physiol.* **34**, 128.
- Crane, F. L. (1961). *Ciba Found. Symp.: Quinones in Electron Transport*, p. 36.
- Crane, F. L. & Lester, R. L. (1958). *Plant Physiol.* **33**, suppl. 7.
- Gloor, U., Isler, O., Morton, R. A., Regg, R. & Wiss, O. (1958). *Helv. chim. acta*, **41**, 2357.
- Gloor, U. & Wiss, O. (1960). *Biochem. biophys. Res. Commun.* **8**, 294.
- Green, D. E. (1961). *Ciba Found. Symp.: Quinones in Electron Transport*, p. 130.
- Hemming, F. W., Pennock, J. F. & Morton, R. A. (1958). *Biochem. J.* **68**, 29 f.
- Kegel, L. P., Henninger, M. D. & Crane, F. L. (1962). *Biochem. biophys. Res. Commun.* **8**, 294.
- Kofler, M. (1946). *Festschr. Emil Borell*, p. 199.
- Kofler, M., Langemann, A., Regg, R., Chopard-dit-Jean, L. H., Rayroud, A., & Isler, O. (1959). *Helv. chim. acta*, **42**, 1283.
- Lawson, D. E. M., Threlfall, D. R., Glover, J. & Morton, R. A. (1961). *Biochem. J.* **79**, 201.
- Lester, R. L., Crane, F. L. & Hatefi, Y. (1958). *J. Amer. chem. Soc.* **80**, 4751.
- Mayer, A. M. & Friend, J. (1960). *J. exp. Bot.* **11**, 141.
- Pennock, J. F., Hemming, F. W. & Morton, R. A. (1960). *Nature, Lond.*, **186**, 470.
- Pennock, J. F., Hemming, F. W. & Morton, R. A. (1962). *Biochem. J.* **82**, 11 f.
- Pennock, J. F., Hemming, F. W. & Morton, R. A. (1963). *Proc. Roy. Soc. (in the Press)*.
- Ramasarma, T. (1961). *J. sci. industr. Res. A*, **20**, 492.
- Reid, W. W. (1961). *Chem. & Ind.* p. 1489.
- Rowland, R. L. & Latimer, P. H. (1959). *Tob. Sci.* **3**, 1.
- Rowland, R. L., Latimer, P. H. & Giles, J. A. (1956). *J. Amer. chem. Soc.* **78**, 4680.
- Seshadri-Sastry, P., Jayaraman, J. & Ramasarma, T. (1961). *Nature, Lond.*, **189**, 577.
- Stoffel, W. & Martius, C. (1960). *Biochem. Z.* **333**, 440.
- Vishniac, W. (1957). In *Methods in Enzymology*, vol. 4, p. 342. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

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The Occurrence of a Long-Chain Ether Analogue of Phosphatidylethanolamine in Brain Tissue

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It has long been known that brain and some other animal tissues contain phospholipids whose phosphorus is not rendered water-soluble by mild alkaline and acid hydrolysis and which are not

sphingomyelins (Brant, 1949; Dawson, 1954; Edgar & Smits, 1959). One of these components has recently been shown to be an artifact, a cyclic acetal of glycerylphosphorylethanolamine pro-

duced by the action of dilute acids on the lysoplasmalogen (Davenport & Dawson, 1962; Pietruszko & Gray, 1962). When the phospholipid mixture surviving mild alkaline and acid treatment is further hydrolysed with hot methanolic hydrochloric acid, the sphingomyelins yield a mixture of phosphorylcholine and sphingosylphosphorylcholine (Dawson, 1958) whilst the cyclic acetal yields glycerophosphate (Ansell & Spanner, 1961*a*, *b*; Davenport & Dawson, 1962). The remaining phosphorus in this fraction is still in lipid form, however, and can be extracted into chloroform or ether (Ansell & Spanner, 1961*a*; Dawson, Hemington & Davenport, 1962).

In a brief report (Ansell & Spanner, 1961*b*) evidence was presented that this chloroform-soluble phospholipid has properties very similar to those of a deacylated long-chain ether phospholipid (glycerol 1-alkoxy-2-acyl-3-phosphorylethanolamine). The natural occurrence of higher glycerol ethers, e.g. batyl alcohol (glycerol 1-hexadecyl ether) and chimyl alcohol (glycerol 1-octadecyl ether) has been known for a long time (see Karnovsky & Brumm, 1955, for references). Carter, Smith & Jones (1958), however, were the first to show the natural occurrence of phosphate esters of long-chain glycerol ethers and they isolated a phosphorylethanolamine derivative of batyl alcohol from egg yolk (I).

Svennerholm & Thorin (1960) briefly reported the separation of a similar lipid from ox brain, and Hanahan & Watts (1961) have isolated a glycerol 1-alkoxy-2-acyl-3-phosphorylethanolamine from ox erythrocytes. Judged from its stability to methanolic hydrochloric acid and hot potassium hydroxide it would appear that such a phospholipid may be a component of spermatozoa (Dr J. Olley, personal communication; Lovern, Olley, Hartree & Mann, 1957).

METHODS

Extraction of lipids from tissues. Total lipids were extracted from fresh tissues by homogenizing at room temperature in 20 vol. of chloroform-methanol (2:1, v/v) and filtering the extract (Folch, Lees & Sloane-Stanley, 1957). Protein-lipid linkages were disrupted by repeated evaporation to dryness (Folch, Ascoli, Lees, Meath & Le Baron, 1951). The lipids were then redissolved in chloroform-methanol (2:1, v/v) and washed by the method of Folch *et al.* (1957).

Materials for column chromatography. Silicic acid (325 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), prepared by the method of Hirsch & Ahrens (1958), was activated by heating overnight at 116°. Aluminium oxide ('For chromatographic analysis') was a product of British Drug Houses Ltd. Florisil (a synthetic magnesia-silica gel absorbant), a product of Floridin Co., was supplied by Hopkin and Williams Ltd. Both the alumina and Florisil were used as supplied.

Chromatography on silicic acid-treated paper. Whatman no. 1 paper was impregnated with silicic acid as described by Marinetti & Stotz (1956), except that 0.1% of soluble starch was added to the sodium silicate solution to prevent powdering of the silicic acid. An ascending technique was used with the solvents of Marinetti & Stotz (1956).

Saponification of lipids. Washed lipids were saponified as described by Ansell & Spanner (1961*a*), except that HCl was always used to neutralize the alkali before extraction of the mild acid- and alkali-stable lipids into CHCl_3 (trichloroacetic acid dissolved in the CHCl_3 if used for the neutralization).

Hydrolysis of mild acid- and alkali-stable fraction. The CHCl_3 solution was taken to dryness and hydrolysed with methanolic HCl as described by Dawson (1958) and Ansell & Spanner (1961*a*). After partition between CHCl_3 and methanol-water the CHCl_3 layer (fraction A) was used for the experiments described in the preliminary studies.

Determination of phosphorus. Phosphorus was usually estimated by the method of Fiske & Subbarow (1925) after oxidation of lipid samples with perchloric acid. Where the amounts of phosphorus were < 10 μg , the method described by Ernster, Zetterström & Lindberg (1950) was used. Phosphorus-containing substances on paper chromatograms, detected by the method of Hanes & Isherwood (1949), were digested with perchloric acid before determination of the orthophosphate content. Determinations of ^{32}P were carried out with conventional liquid-counting equipment.

Nitrogen determination. An amount of material considered to contain 50 μg of N was digested in a micro-Kjeldahl flask, the NH_3 was distilled into 1 ml. of 0.1N-HCl in a Conway microdiffusion apparatus and estimated with Nessler's reagent (Weil-Malherbe & Green, 1955).

Determination of ethanolamine. After acid hydrolysis of lipids in methanolic 5.8N-HCl under reflux for 3 hr., water-soluble ethanolamine was estimated by the method of Appleton, La Du, Levy, Steel & Brodie (1953).

Glycerol ethers. When both the primary and secondary hydroxyl groups of 1-alkoxy-glycerols were exposed it was possible to determine the glycerol ether essentially by the method of Karnovsky & Brumm (1955). After hydrolysis of the lipid (see Results section) an amount of CHCl_3 -soluble material, equivalent to about 0.75 μmole of batyl alcohol, was evaporated to dryness, dissolved in 0.25 ml. of 95% ethanol and 0.25 ml. of periodic acid was added (1% solution of the dihydrate in 95% ethanol). After 60 min. at room temperature, 0.75 ml. of water, 0.375 ml. of N-HCl and 0.125 ml. of 1.2N-arsenite were added and the solution was diluted to 2.5 ml. A sample (0.5 ml.) was taken for the determination of formaldehyde with chromotropic acid. Batyl alcohol (L. Light and Co. Ltd., Colnbrook, Bucks) was used as a standard. When acetolysis had been employed the material soluble in diethyl ether was evaporated to dryness, saponified overnight at room temperature

in ethanolic *N*-KOH and the free glycerol ether was re-extracted into diethyl ether after dilution of the ethanolic alkali with water. The solution of the glycerol ether was then evaporated to dryness and oxidized as described above.

RESULTS

Preliminary studies. Prolonged hydrolysis of the chloroform-soluble material (fraction A) in aqueous 2*N*-hydrochloric acid for 48 hr. at 123° yielded some inorganic phosphate and two water-soluble ninhydrin-reacting compounds; one of these was judged to be ethanolamine by chromatography with a number of solvents including anhydrous pyridine (Brady & Koval, 1958). The other compound remained at the origin in phenol–butan-1-ol–formic acid–aq. potassium chloride (Bremer, Figard & Greenberg, 1960) and ran rather slower than phosphorylethanolamine in phenol–water–aq. 18*N*-ammonia solution (80:20:0.3, w/v/v).

The only known phospholipids whose phosphorus would survive in a lipid-bound form to the stage of fraction A are the ether analogues of diacylphospholipids. Through the kindness of Dr T. H. Bevan of Bristol University we obtained chimyl (glycerol 1-hexadecyl ether) and batyl (glycerol 1-octadecyl ether) analogues of phosphatidylethanolamine esterified in the 2-position with stearic acid (Baylis, Bevan & Malkin, 1958). When these were saponified as described by Ansell & Spanner (1961*b*) most of the phosphorus remained soluble in chloroform after acidification and the recovery could be improved by back-washing (Table 1). Hydrolysis of the deacylated material with methanolic 2*N*-hydrochloric acid for 1.5 hr. at 100° yielded no water-soluble phosphorus when the hydrolysate was shaken with chloroform–water. Although the ethanolamine remained attached to the lipid during saponification (Carter *et al.* 1958)

it was expected that this base would be liberated during the hot-acid hydrolysis. It has been shown by Klenk & Debuch (1954) and Debuch (1959) that when the glycerol 1-alkoxy-2-acyl-3-phosphorylethanolamine, obtained on hydrogenation of the ethanolamine plasmalogen, is hydrolysed with methanolic 0.8–1.2*N*-hydrochloric acid under reflux, the ethanolamine is completely liberated. Ethanolamine was not, however, completely removed by hydrolysis in a sealed tube in the present experiments. When the chloroform-soluble material obtained after this hydrolysis was hydrolysed again for 48 hr. in aq. 2*N*-hydrochloric acid at 123° and the water-soluble material, remaining after re-extraction with chloroform, was run on paper chromatograms, two ninhydrin-reacting spots were obtained. One of these was ethanolamine and the other behaved exactly as did the unknown obtained from the brain lipid. This behaviour of both synthetic and brain lipid suggested that they were very similar.

Chromatography on silicic acid. An attempt was made to purify material A on a column of silicic acid (2 g.). On applying the material in chloroform solution in the conventional fashion all the phosphorus was retained whilst material judged to be the methyl esters of fatty acids and dimethylacetals of long-chain fatty aldehydes passed through. When the column was eluted with chloroform–methanol (4:1, v/v), between 40 and 70 % of the phosphorus originally applied to the column was eluted (Table 1). A similar result was obtained with synthetic analogues, saponified and hydrolysed beforehand. The eluted material obtained on chromatography of the synthetic phospholipid had the ratio nitrogen:phosphorus 1:1 but the material from brain had nitrogen:phosphorus in excess of 18:1, and although this eluted material ran as a single component on silicic acid papers it was

Table 1. *Behaviour of synthetic alkyl ether phospholipids and the phospholipid from brain towards hydrolytic procedures and on silicic acid columns*

For details of the experimental procedure see the text.

	Phospholipid stable to treatment with mild alkali and acid (%)	Mild alkali- and acid-stable phospholipid, soluble in chloroform after hydrolysis in hot methanolic hydrochloric acid (%)	Chloroform-soluble fraction eluted from silicic acid column with chloroform-methanol (4:1, v/v) (%)
Ox-brain corpus callosum	—	—	51
Rat whole brain	—	—	70
Synthetic batyl analogue	—	100	37
	69*	100	53
	75*	100	38
	—	97	51
Synthetic chimyl analogue	100	100	58

* A further 6–10 % could be obtained by re-extracting the aqueous methanol phase with more chloroform.

obviously impure. The nitrogenous contaminant was probably sphingosine, since this is known to be partially eluted from silicic acid columns with chloroform-methanol (4:1, v/v) (Brady, Formica & Koval, 1958). Furthermore, after hydrolysis of this material for 48 hr. in 2*N*-hydrochloric acid the chloroform-soluble product yielded formaldehyde in the method of Karnovsky & Brumm (1955) far in excess of any free glycerol ether that could be present. All the chloroform-methanol (4:1, v/v) eluates from synthetic or brain material yielded the two characteristic water-soluble ninhydrin-reacting substances on prolonged hydrolysis.

Isotope-dilution experiment. The similarity between the deacylated batyl (or chimyl) analogue and the brain lipid was further demonstrated by the following experiment.

Two rats (each 78 g. body wt., 4 weeks old) were injected intraperitoneally with 12 μ C of [32 P]orthophosphate/g. body wt. After an exchange period of 24 hr. the animals were killed, the total brain lipids were pooled and then divided into two equal parts. To one sample was added 16 mg. of the synthetic batyl analogue ($\equiv$ 750 μ g. of phosphorus) and both samples were then taken through the saponification and acid-hydrolysis procedure. After hydrolysis with methanolic hydrochloric acid the control contained 57.7 μ g. of phosphorus in fraction A and the diluted sample 508 μ g. of phosphorus (the loss of about 40% of added analogue in this experiment could not be explained).

Both samples were then subjected to chromatography on silicic acid columns and both 31 P and 32 P were determined in the samples as they were eluted (Fig. 1). There was good coincidence of radioactivity and added phosphorus in both the main peak eluted with chloroform-methanol (4:1, v/v) and the subsequent smaller peak eluted with chloroform-methanol (1:1, v/v). A similar pattern was obtained with the untreated sample; the slightly slower elution of the main peak in this instance might be due to the fact that much less material was applied to the column. Between 70 and 80% of the phosphorus and radioactivity applied to the column was recovered in each instance. When a fraction of the main peak of the treated sample was run in di-*n*-butyl ether-chloroform-acetic acid-water on silicic acid papers (Marinetti & Stotz, 1956), a single ninhydrin-reacting, radioactive lipid was obtained, though there were one or two very faint additional radioactive spots.

Chromatography of brain lipids on alumina. Since the hydrolysis with methanolic 2*N*-hydrochloric acid removed a variable amount of ethanolamine it was decided to adopt a different approach in an attempt to obtain the ether lipid by a milder procedure that would also eliminate most of the

sphingolipids at an earlier stage in the separation. For this purpose the chromatographic separation on alumina developed by Rhodes & Lea (1957), Long & Staples (1961*a, b*) and Davison & Wajda (1959, 1962) seemed appropriate.

Preparation 1. The total lipids of ox-brain corpus callosum (13.9 g. wet wt.) were extracted as described above and the washed lipids applied to a 60 g. (28 cm. $\times$ 2 cm.) column of alumina in chloroform-methanol (1:1, v/v). Choline-containing lipids and cholesterol passed straight through the

Fig. 1. Chromatography of the radioactive brain-lipid fraction A on silicic acid columns. (a) Brain lipid alone. Total phosphorus and radioactivity applied to column, 52.2 μ g. and 1525 counts/min. (b) Brain lipid to which the synthetic batyl analogue had been added before saponification. Total phosphorus and radioactivity added to column, 458 μ g. and 1608 counts/min. Eluents: (1) chloroform; (2)-(4) chloroform-methanol, 4:1 (v/v), 1:1 (v/v), 1:4 (v/v) respectively. See text for details. $\blacktriangle$, Phosphorus (μ g.); $\bullet$, 32 P.

column and it can be seen from Table 2 that the bulk of the ethanolamine-containing lipid was obtained with chloroform-ethanol-water (2:5:2, by vol.) (cf. Davison & Wajda, 1962). After saponification and mild acid treatment alone, 36% of the phosphorus was found to be chloroform-soluble. When this alkali-stable lipid was further hydrolysed with methanolic hydrochloric acid, 4-5% of the lipid phosphorus, calculated as a percentage of the original eluate, behaved as an ether lipid in that it was still chloroform-soluble. The cyclic acetal of glycerylphosphorylethanolamine (Davenport & Dawson, 1962), which yielded glycerophosphate and ethanolamine on hot-acid hydrolysis, accounted for the difference between the two values. To eliminate the cyclic acetal without removing any ethanolamine from the ether lipid the fraction stable to mild alkali and acid was heated for 10 min. at 60° in methanolic *N*-hydrochloric acid (Ansell & Norman, 1956), the reaction stopped by cooling the mixture to 0° and the remaining lipid phosphorus extracted into chloroform by shaking with chloroform-water. (The water-soluble fraction consisted of glycerylphosphorylethanolamine and a trace of ethanolamine.) To eliminate methyl esters of fatty acids and dimethylacetals of aldehydes from the lipid mixture the chloroform layer was evaporated to dryness, redissolved in 10 ml. of chloroform and applied to a 3 g. column of silicic acid. After washing the column with a further 30 ml. of chloroform the phospholipid was eluted quantitatively with 50 ml. of chloroform-methanol (4:1, v/v). It moved as a single ninhydrin-reacting, phosphorus-containing spot in di-*n*-butyl ether-acetic acid-chloroform-water (Marinetti & Stotz, 1956) to the same position as the saponified batyl analogue. The ratio N:P was 1:23:1, however, suggesting some contamination.

Preparation 2. A further sample from ox-brain corpus callosum was prepared and this had the ratio N:P 1:43:1. It was thought likely that the contaminant was a sphingolipid, i.e. a sulphatide that is known to be partially eluted with the ethanolamine lipids from an alumina column (Long & Staples, 1961*b*). To purify the lipid the material (0.97 mg. of phosphorus) was dissolved in 10 ml. of chloroform-methanol (2:1, v/v) and run through a 3 g. column of Florisil suspended in the same solvent. The column was washed with a further 30 ml. of the solvent to remove sphingolipids; the phosphorus was completely adsorbed. Phospholipid was then quantitatively eluted with the same solvent saturated with water (Lees, Folch, Sloane-Stanley & Carr, 1959; Svennerholm & Thorin, 1960). This phospholipid had the ratio N:P 1:1:1. After hydrolysis with methanolic 5-8*N*-hydrochloric acid under reflux for 3 hr. the water-soluble ethanolamine released gave the ratio phosphorus:ethanolamine 1:0.84.

The yield of this lipid was equivalent to about 87 μ g. of lipid phosphorus/g. of white matter or 3-4% of the lipid phosphorus. Since the total amount of ether phospholipids in ox-brain white matter was found to be no more than 100 μ g. of phosphorus/g. (Ansell & Spanner, 1961*a*), it is highly likely that this ethanolamine lipid is the major component.

Chromatography of egg-yolk lipid. Since Carter *et al.* (1958) obtained their ether lipid from egg yolk it seemed advisable to see if the isolation procedure described above yielded comparable material. Accordingly 15.5 g. of frozen egg yolk was extracted by the usual procedure and the total washed lipid fraction in chloroform-methanol (1:1, v/v) was applied to a 60 g. column of alumina. When the fraction eluted with chloroform-ethanol-water

Table 2. *Fractionation of the total lipids from 23.9 g. of ox-brain corpus callosum*

The total lipids were applied to the alumina column as described in the text. Different fractions were eluted as indicated below in a stepwise fashion.

Eluent	Vol. (ml.)	Product	Remarks
Chloroform-methanol (1:1, v/v)	400	Choline-containing lipids and cholesterol	Discarded
Chloroform-methanol-water (7:7:1, by vol.)	300	Largely cerebroside	Discarded
Chloroform-methanol-water (2:5:1, by vol.)	500	Phospholipid $\equiv$ 1.74 mg. of P	Small amount of ethanolamine phospholipids present. Hydrolysis of the alkali-stable fraction in methanolic 2 <i>N</i> -hydrochloric acid yielded no chloroform-soluble P
Chloroform-ethanol-water (2:5:2, by vol.)	270	Phospholipid $\equiv$ 11.69 mg. of P	Largely ethanolamine lipids. Hydrolysis of alkali-stable fraction in methanolic 2 <i>N</i> -hydrochloric acid yielded 4-5% of chloroform-soluble P
Chloroform-ethanol-water (2:5:2, by vol.)	270	Phospholipid $\equiv$ 0.45 mg. of P	Discarded

Table 3. *Cleavage of natural and synthetic long-chain ether analogues of phosphatidylethanolamine*

Hydrolyses with hydrochloric acid were carried out in sealed tubes. Hydrolyses with acetic acid-acetic anhydride were carried out under reflux.

Expt. no.	Lipid source	P:N:ethanol-amine	Method of cleavage	Total P (μmole)	Water-soluble P (μmole)	Water-soluble N (μmole)	Batyl alcohol (μmole)
1	Ox-brain corpus callosum (preparation 1)	1:1:21:-	2N-HCl in methanol (68 hr. at 123°)	1	0.53	—	0.52
2	Ox-brain corpus callosum (preparation 1)	1:1:21:-	2N-HCl in methanol-water, 1:1, v/v (47 hr. at 123°)	1	0.61	—	0.86
3	Saponified batyl analogue	1:-:1:03	2N-HCl in methanol-water, 1:1, v/v (47 hr. at 123°)	1	0.40	—	0.39
4	Saponified batyl analogue	1:-:1:03	2N-HCl in methanol-water, 1:1, v/v (24 hr. at 110°)	1	0.26	0.93	0.22
5	Saponified batyl analogue	1:-:1:03	Acetolysis (8.5 hr.)	1	0.40†	0.92	0.37
6	Ox-brain corpus callosum (preparation 1)	1:1:21:-	Acetolysis (8.5 hr.)	1	0.15†	0.90	0.14
7	Ox-brain corpus callosum* (preparation 2)	1:1:1:0.84	Acetolysis (8.5 hr.)	1	0.73†	0.70	0.19
8	Egg yolk*	1:0.87:0.89	Acetolysis (8.5 hr.)	1	0.55‡	0.58	0.15

* Purified with Florisil. † Inorganic phosphate (total phosphate not measured).
‡ Inorganic phosphate < 0.1 μmole.

(2:5:2, by vol.) was saponified and the slightly acidified suspension extracted with methanol-chloroform as described above, the alkali-stable lipid represented only 6% of this fraction. Since this 6% was stable to methanolic *n*-hydrochloric acid at 60° for 10 min. the observation by Rhodes & Lea (1957) and Thiele (1955) that egg yolk contains little or no ethanolamine plasmalogen is confirmed. When this lipid was adsorbed on Florisil from methanol-chloroform (2:1, v/v) and eluted with the same solvent saturated with water it had the proportions phosphorus:nitrogen:ethanol-amine 1:1:0.48, suggesting the presence of another nitrogen-containing phospholipid. Adsorption on silicic acid from pure chloroform and elution with chloroform-methanol (4:1, v/v) yielded a lipid with proportions phosphorus:nitrogen:ethanol-amine 1:0.87:0.89. This lipid behaved as the ox lipid and the saponified batyl analogue on silicic acid paper in di-*n*-butyl ether-chloroform-acetic acid. There was sometimes a faster-moving ninhydrin-negative spot that could have been batyl phosphate.

Hydrolysis and acetolysis of synthetic lipid and the lipid from ox brain and egg yolk. In Table 3 are shown the yields of components of the ethanol-amine phospholipid after various methods of cleavage. It will be seen that the prolonged hydrolysis with 2N-hydrochloric acid in anhydrous or aqueous methanol yielded only about half the phosphorus of the brain lipid as a water-soluble component. The yield from the batyl analogue was rather less. In both instances the free batyl alcohol obtained was equivalent to the phosphorus re-

Fig. 2. Hydrolysis of saponified batyl analogue and ox-brain lipid (preparation 1) in 2N-hydrochloric acid in methanol-water (1:1, v/v) at 123°. Brain: ▲, water-soluble phosphorus; △, inorganic phosphate. Batyl analogue: ●, water-soluble phosphorus; ○, inorganic phosphate.

leased. In Fig. 2 is shown the rate of release of total water-soluble phosphorus and inorganic phosphate from both lipids in 2N-hydrochloric acid in aqueous methanol. Although the rate of inorganic phosphate release was similar in both instances the release of total water-soluble phosphorus from the brain lipid was more rapid in the earlier stages of the hydrolysis. A similar result, but with a slower overall rate of cleavage, was obtained with anhydrous methanolic hydrochloric acid. From an examination of paper chromatato-

grams of the hydrolysates it was considered that the difference between the inorganic phosphate and the total water-soluble phosphorus was represented by phosphorylethanolamine. This ester is known to be released from ethanolamine-containing glycerophospholipids on hydrolysis with acid (Dittmer, Feminella & Hanahan, 1958) and the same would appear true for the long-chain ether analogues.

Acetolysis was carried out with acetic anhydride-acetic acid (1:4, v/v) for 8.5 hr. at the boiling point as described by Bevan, Brown, Gregory & Malkin (1953). Water- and diethyl ether-soluble fractions were obtained as described by Carter *et al.* (1958). This method released a variable amount of water-soluble phosphorus and a variable amount of nitrogen (Table 3), whereas prolonged treatment with methanolic hydrochloric acid usually converted all the nitrogen into a water-soluble form. Sometimes very little inorganic phosphate was obtained (Table 3), which would imply that the phosphorus was in a water-soluble organic linkage, presumably as phosphorylethanolamine. The batyl alcohol determinations in these experiments were not always satisfactory, particularly with egg yolk (Table 3).

According to Bevan & Malkin (1960) the α -batyl analogue of phosphatidylethanolamine is completely dephosphorylated on acetolysis, the β -analogue less so. The lipid from egg yolk obtained by Carter *et al.* (1958) yielded some phosphorylethanolamine but the extent of the dephosphorylation was not stated.

The implication of these experiments is that the lipid from brain and egg yolk is extremely similar to the synthetic material. Differences in behaviour may be due to varying degrees of purity.

Infrared spectra. Approximately 2 mg. (calculated) of the lipid obtained from ox-brain corpus callosum (preparation 2) in chloroform-methanol was evaporated to dryness in the presence of 300 mg. of potassium bromide and its infrared spectrum was compared with that of the saponified batyl analogue. There was a very close correlation and the following peaks were obtained from both: 3520 (amino and hydroxyl groups), 2980 and 2900 (hydrocarbon-stretching frequency), 1480 (hydrocarbon-deformation frequency), 1230 (P = O, hydrogen bonding?), 1080 (alkyl ether group + phosphate ester group), 800 and 755 (amino group), 722 cm^{-1} (long-chain hydrocarbon). In both instances there was evidence of some contamination with fatty acid (1750 and 1650 cm^{-1}). The infrared spectrum of a glycerol 1-alkoxy-3-phosphorylethanolamine (prepared from ethanolamine plasmalogen) shown to us by Dr G. M. Gray was also very similar to those given by the brain lipid and synthetic lipid obtained in these experiments.

DISCUSSION

From the observations described in this paper it is highly likely that the phospholipid surviving alkaline and acid hydrolysis followed by a more vigorous hydrolysis with hot methanolic hydrochloric acid is a derivative of a glycerol 1-alkoxy-3-phosphate or a mixture of such phosphates. Svennerholm & Thorin (1960) have also produced evidence that such an ethanolamine lipid exists in human and ox brain to the extent of 2% of the lipid phosphorus. This value agrees with that more recently obtained by Dawson, Hemington & Davenport (1962) for the total alkyl ether lipid in ox brain and that for rat brain given by Ansell & Spanner (1961b).

It is difficult to prove that a long-chain ether analogue of phosphatidylethanolamine is not an artifact derived from the reduction of the vinyl ether linkage of the ethanolamine plasmalogen. This plasmalogen is present in large amounts in brain tissue, particularly white matter (Webster, 1960). On the other hand the saturated ether lipid has been found in liver (Svennerholm & Thorin, 1960) which contains very little ethanolamine plasmalogen (Klenk & Friedrichs, 1952; Klenk & Dohmen, 1955; Dawson *et al.* 1962). In addition Pietruszko & Gray (1962) have shown that glycerol ethers cannot be produced as a side reaction in the deacylation of plasmalogens. Carter *et al.* (1958) and Svennerholm & Thorin (1960) are of the opinion that the long-chain ether analogues of phosphatidylethanolamine are acylated in the native state. Pietruszko & Gray (1962) have demonstrated that, after removal of the aldehyde from a mixture of (largely) phosphatidylethanolamine and ethanolamine plasmalogen obtained from ox heart, the glycerol ether appeared, not in the lysophosphatidylethanolamine fraction, but in the fraction containing diacyl phospholipids. This strongly suggests that the native ether lipids are acylated. More definite evidence for the presence of the acyl group has been presented by Hanahan & Watts (1961) from the results of experiments on a mixture of the diacylphospholipid and ether lipid from ox erythrocytes. After a short hydrolysis with 0.5N-sodium hydroxide (3 min. at 10°) the diacylphospholipid was completely deacylated whereas the fatty acid from the ether lipid was removed to only a limited extent. The glycerol 1-alkoxy-2-acyl-3-phosphorylethanolamine could be separated from its deacylated product on a silicic acid column.

It has been consistently observed in the experiments described in this paper that the deacylated long-chain ether analogue prepared from the synthetic acylated lipid or the brain is eluted from a silicic acid column with chloroform-methanol

(4:1, v/v); other workers have found much higher concentrations of methanol to be essential. Thus Carter *et al.* (1958) found concentrations of methanol in excess of 30 % (v/v) necessary to elute the deacylated lipid, and Hanahan & Watts (1961) used chloroform-methanol (1:4, v/v). Davenport & Dawson (1962), using a gradient of methanol in chloroform to separate various components in a mixture of mild acid- and alkali-stable phospholipids from ox heart, obtained two distinct peaks which contained lipid-bound phosphorus surviving hydrolysis in methanolic hydrochloric acid at 100°. For heart tissue the situation is complicated because ether phospholipids containing choline as well as those containing ethanolamine are present (Pietruszko & Gray, 1962). If the deacylated ethanolamine ether lipid were eluted with the cyclic acetal ethanolamine lipid in the experiments of Davenport & Dawson (1962), as seems likely, then a methanol concentration of 50–60 % (v/v) was required. On the other hand, the other peak was eluted with chloroform-methanol (4:1, v/v).

There are other discrepancies in connexion with the cleavage of the lipid either with hydrochloric acid or with acetic acid-acetic anhydride. It was observed that the deacylated ether lipid behaved in many respects like the synthetic material. Although the results shown in Table 3 illustrate this similarity it must be conceded that the release of total phosphorus from the brain material proceeded at a faster rate than that from the synthetic lipid (Fig. 2). It was, however, slow in both instances. There are very few reports on the prolonged acid hydrolysis of phospholipids of this type. Short-term hydrolysis with methanolic hydrochloric acid tends to release only ethanolamine (Debuch, 1959). Marinetti & Erbland (1957), like Debuch (1959), worked with a hydrogenated product from plasmalogen, though one containing choline. They showed that the release of inorganic phosphate from the glycerol 1-alkoxy-3-phosphate finally obtained amounted to only 35 % in 24 hr. in 6N-sulphuric acid. The relative stability of the phosphate ester linkage is what one might expect by analogy with glycerol 3-phosphate. Stegerhoek & Verkade (1956), on the other hand, claimed that the synthetic batyl 1-phosphate prepared by them was very unstable, yielding inorganic phosphate spontaneously even when stored over phosphorus pentoxide. This anomalous observation obviously requires confirmation.

The results of the acetolysis of the synthetic and native lipid were very similar but complete cleavage to water-soluble phosphorus and nitrogen was not obtained. Quantitative information on this particular method is scarce, though in their original publication Bevan *et al.* (1953) obtained nearly quantitative replacement of the phosphate

group by an acetyl group for a number of synthetic phospholipids. Bevan & Malkin (1960) later observed that the synthetic batyl analogue of phosphatidylethanolamine (glycerol 1-hexadecoxy-2-stearoyl-3-phosphorylethanolamine) gave an 86 % yield of the 1-acetate-2-stearate. A synthetic lipid with the ether group in the 2-position (β -batyl or 2-hexadecyl ether) gave a product that still contained nitrogen and phosphorus. Carter *et al.* (1958) obtained α -batyl diacetate (glycerol 1-hexadecoxy-2,3-diacetate) by acetolysis of their deacylated egg-yolk lipid and presumably no diethyl ether-soluble phosphorus remained. After acetolysis of a mixture of phosphatidylethanolamine (23 %) and its batyl analogue (77 %) from ox erythrocytes, Hanahan & Watts (1961) found that the ether extract contained no phosphorus and the yield of batyl alcohol on saponification was 80 %. Very little information is available on the form in which the phosphorus is liberated. Carter *et al.* (1958) found a little phosphorylethanolamine and large amounts of inorganic phosphate. In many of the experiments described in this paper the yield of inorganic phosphate was extremely small and it has been assumed that the remainder of the water-soluble phosphorus was phosphorylethanolamine.

SUMMARY

1. Evidence is presented for the occurrence of a long-chain ether analogue of phosphatidylethanolamine in brain tissue.

2. The deacylated alkyl ether phospholipid can be obtained in small amounts from the white matter of brain tissue by a combination of selective hydrolysis and chromatography on alumina, silicic acid and Florisil.

3. It is estimated that the alkyl ether phospholipid does not account for more than 2–3 % of the phospholipid in whole rat brain or 3 % in ox-brain white matter.

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REFERENCES

- Ansell, G. B. & Norman, J. N. (1956). *J. Neurochem.* **1**, 32.
Ansell, G. B. & Spanner, S. (1961a). *Biochem. J.* **79**, 176.
Ansell, G. B. & Spanner, S. (1961b). *Biochem. J.* **81**, 36P.
Appleton, H. D., La Du, B. N., Levy, B. B., Steel, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.

- Baylis, R. L., Bevan, T. H. & Malkin, T. (1958). *J. chem. Soc.* p. 2962.
- Bevan, T. H., Brown, D. A., Gregory, G. I. & Malkin, T. (1953). *J. chem. Soc.* p. 127.
- Bevan, T. H. & Malkin, T. (1960). *J. chem. Soc.* p. 350.
- Brady, R. O., Formica, J. V. & Koval, G. J. (1958). *J. biol. Chem.* **233**, 1072.
- Brady, R. O. & Koval, G. J. (1958). *J. biol. Chem.* **233**, 26.
- Branté, G. (1949). *Acta physiol. scand.* **18**, suppl. 63.
- Bremer, J., Figard, P. H. & Greenberg, D. M. (1960). *Biochim. biophys. Acta*, **43**, 477.
- Carter, H. E., Smith, D. B. & Jones, D. N. (1958). *J. biol. Chem.* **232**, 681.
- Davenport, J. B. & Dawson, R. M. C. (1962). *Biochem. J.* **84**, 490.
- Davison, A. N. & Wajda, M. (1959). *J. Neurochem.* **4**, 353.
- Davison, A. N. & Wajda, M. (1962). *Biochem. J.* **82**, 113.
- Dawson, R. M. C. (1954). *Biochem. J.* **56**, 621.
- Dawson, R. M. C. (1958). *Biochem. J.* **68**, 357.
- Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
- Debuch, H. (1959). *Hoppe-Seyl. Z.* **317**, 182.
- Dittner, J. C., Feminella, J. L. & Hanahan, D. J. (1958). *J. biol. Chem.* **233**, 862.
- Edgar, G. W. F. & Smits, G. (1959). *J. Neurochem.* **3**, 316.
- Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
- Folch, J., Lees, M. & Sloane-Stanley, G. A. (1957). *J. biol. Chem.* **226**, 497.
- Hanahan, D. J. & Watts, R. (1961). *J. biol. Chem.* **236**, rc59.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hirsch, J. & Ahrens, E. H., jun. (1958). *J. biol. Chem.* **233**, 311.
- Karnovsky, M. L. & Brumm, A. F. (1955). *J. biol. Chem.* **216**, 689.
- Klenk, E. & Debuch, H. (1954). *Hoppe-Seyl. Z.* **296**, 179.
- Klenk, E. & Dohmen, H. (1955). *Hoppe-Seyl. Z.* **299**, 248.
- Klenk, E. & Friedrichs, E. (1952). *Hoppe-Seyl. Z.* **290**, 169.
- Lees, M., Folch, J., Sloane-Stanley, G. H. & Carr, S. (1959). *J. Neurochem.* **4**, 9.
- Long, C. & Staples, D. A. (1961a). *Biochem. J.* **78**, 179.
- Long, C. & Staples, D. A. (1961b). *Biochem. J.* **80**, 557.
- Lovern, J. A., Olley, J., Hartree, E. F. & Mann, T. (1957). *Biochem. J.* **67**, 630.
- Marinetti, G. V. & Erbland, J. (1957). *Biochim. biophys. Acta*, **26**, 429.
- Marinetti, G. V. & Stotz, E. (1956). *Biochim. biophys. Acta*, **21**, 168.
- Pietruszko, R. & Gray, G. M. (1962). *Biochim. biophys. Acta*, **56**, 232.
- Rhodes, D. N. & Lea, C. H. (1957). *Biochem. J.* **65**, 526.
- Stegerhoeck, L. J. & Verkade, P. E. (1956). *Rec. Trav. chim. Pays-Bas*, **75**, 467.
- Svennerholm, L. & Thorin, H. (1960). *Biochim. biophys. Acta*, **41**, 371.
- Thiele, O. W. (1955). *Hoppe-Seyl. Z.* **299**, 151.
- Webster, G. R. (1960). *Biochim. biophys. Acta*, **44**, 109.
- Weil-Malherbe, H. & Green, R. H. (1955). *Biochem. J.* **61**, 210.

Biochem. J. (1963) **88**, 64

Biochemistry of Dystrophic Muscle

2. SOME ENZYME CHANGES IN DYSTROPHIC MOUSE MUSCLE*

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Following upon the discovery (Michelson, Russell & Harman, 1955) of a hereditary myopathy in a strain of mice, several workers have measured the activity of a number of enzymes in muscle from the affected animals. Weinstock, Epstein & Milhorat (1958) found a marked increase in acid-cathepsin activity and in cytochrome oxidase but no significant difference in aldolase or succinate oxidase; these results were obtained when comparison with normal muscle was made on the basis of either non-collagen nitrogen or total nitrogen. On the other hand, White (1959) reported a

decrease of aldolase and of lactate dehydrogenase, when comparison was made on a total protein basis; isocitrate-dehydrogenase activity, on the other hand, was increased. α -Glucan phosphorylase and phosphoglucomutase are decreased when based on fresh weight and also, in the latter case, when based on non-collagen nitrogen (Leonard, 1957; Hazzard & Leonard, 1959). McCaman (1960) found a striking increase (about fourfold) in glucose 6-phosphate dehydrogenase, and smaller increases in two other NADP-linked enzymes, isocitrate dehydrogenase and glutathione reductase (all based on fat-free dry weight); in contrast, lactate dehydrogenase and α -glycerophosphate de-

* Part 1: Pennington (1961a).