as starting material generally moved to the solvent front, while the unsaturated alcohols, being more polar, had lower R_F values.

Apart from providing useful TLC separation procedures, these results also demonstrate that addition of mercaptoacetic acid to monoene fatty acids, esters and alcohols yields both isomers, with the exception of undecenoic acid which gives rise to a single product probably the 11-carboxymethylthioundecanoic acid.

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1 N. H. KOENIG AND D. SWERN, *J. Am. Chem. Soc.*, 79 (1957) 362.
2 S. P. FORE, R. T. O'CONNOR AND L. A. GOLDBLATT, *J. Am. Oil Chemists' Soc.*, 35 (1958) 225.
3 L. R. ESHELMAN, E. Y. MANZO, S. J. MARCUS, A. E. DECOTEAU AND E. G. HAMMOND, *Anal. Chem.*, 32 (1960) 844.
4 M. R. SUBBARAM, M. W. ROOMI AND K. T. ACHAYA, *J. Chromatog.*, 21 (1966) 324.
5 R. SUBBARAO, M. W. ROOMI, M. R. SUBBARAM AND K. T. ACHAYA, *J. Chromatog.*, 9 (1962) 295.
6 M. W. ROOMI, M. R. SUBBARAM AND K. T. ACHAYA, *J. Chromatog.*, 16 (1964) 106.

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Separation and quantitative determination of ^{32}P-labelled lipids from brain particulates by thin-layer chromatography

The separation and isolation of phospholipids by thin-layer chromatography (TLC) on silica gel has been reported by several investigators[1–5]. Although a distinct separation of some phospholipids was achieved by these techniques, they yield separations with overlap among such phospholipids as phosphatidic acid and neutral lipids or phosphatidyl inositol and phosphatidyl serine. During our work on the effect of neurohormones and metabolic inhibitors of phosphate metabolism in nerve-ending particulates of rat brain, it was found that phospholipids were highly labelled when incubated in an oxidative phosphorylation medium containing ^{32}P-orthophosphate[6]. By incorporating certain features of previously described techniques[1,4], nerve endings or mitochondrial ^{32}P-labelled phospholipids, including the highly labelled phosphatidic acid and phosphatidyl inositol, were effectively separated on Silica Gel G by means of two-dimensional TLC, and the radioactive specific activities of the individual phospholipids were determined.

Experimental

Lipid extracts. Total lipids were extracted from rat brain nerve-ending particles or purified mitochondria with chloroform–methanol (2:1) after incubation in an oxidative phosphorylation medium containing 100–150 μc of ^{32}P-orthophosphate for 1 h at 37° as described previously[7]. The extract was filtered, concentrated *in vacuo*

and the lipid concentrate taken up in chloroform. Solutions of lipid in chloroform were adjusted so that 1 ml solution contained 27 μmoles of lipid phosphorus.

Phospholipid standards were products of Pierce Chemical Company, Rockford, Ill.

Preparation of chromatographic plates and solvents. To 40 g of Silica Gel G (Brinkmann Instruments), 80 ml of deionized water were added, and the resultant slurry was mixed with constant stirring for 4 min. Five glass plates, 20 × 20 cm, were coated with this slurry to a depth of 0.25 mm using the Desaga adjustable applicator. The plates were air-dried and activated for 1 h at 110° just before use.

Two different solvent systems were used for the development of the two-dimensional thin-layer chromatograms:

Solvent A: chloroform–methanol–acetic acid–water (25:15:4:2)[1].

Solvent B: butanol–pyridine–water (45:5:20)[4].

Spotting and development. For one-dimensional thin-layer chromatograms, 40–60 μl of lipid extract were applied to the TLC plates with micropipettes, and allowed to develop in equilibrated jars containing Solvent A. The solvent was allowed to rise within 2 cm of the top of the adsorbent. Average running time was 1.1 h. The plates were removed from the chromatography jars, dried for 30 min and placed in Solvent B after clockwise rotation through 90°. Average running time was 3 h.

Detection of spots. Lipids were detected on dried chromatograms in the following manner:

The plates were exposed to iodine vapor[8] and the spots were immediately outlined with the point of a needle. For the detection of phosphatidyl serine, phosphatidyl ethanolamine, and plasmalogens, the plates were sprayed with ninhydrin. Phosphatidic acid was identified, after elution from the thin-layer chromatogram, by deacylation according to the procedure of DAWSON[9]. The radioactive product and standard α-glycerophosphate were run in paper electrophoresis at pH 10.8 as described previously[7]. Other phospholipids of nerve-ending particles or purified mitochondria were identified by chromatographing standard phospholipids and comparison with previously published data using the same solvent system[1]. For the detection of radioactive lipids the chromatoplates were wrapped in Saferan film, and then exposed to Kodak No-Screen X-ray film for 3 days.

Analysis of the ^{32}P-labelled phospholipids. The radioactive phospholipid spots detected by autoradiography, and the non-radioactive lipids detected by iodine vapor, were scraped off with a razor blade and the radioactivity was measured with a gas-flow counter and scaler. After counting, the silica gel from each planchette was poured into test tubes and 0.25 ml of concentrated sulfuric acid was added to all tubes including blank silica gel, reagent blanks and inorganic phosphate standards. The tubes were placed in a heating block and digested according to the procedure of PARKER AND PETERSON[10].

Phospholipid phosphorus was determined by a modification of the methods of KING[11] and BARTLETT[12]. To all the tubes, 4.05 ml of water, 0.5 ml of 5 % ammonium molybdate solution and 0.2 ml of 1-amino-2-naphthol-4-sulfonic acid reagent were added. The latter reagent was prepared according to the method of KING[11]. After heating in a boiling water bath for 10 min, the tubes containing silica gel were centrifuged at 300 × g for 40 min. The color was read at wavelength of 620 mμ (see Fig. 2) on a Beckman Model B Spectrophotometer using glass cells with a 1 cm light path.

Results and discussion

The resolution of mixtures of ^{32}P-labelled phospholipids, extracted either from brain mitochondria or nerve-ending particles was found to be incomplete after running in one-dimensional TLC (Fig. 1A). However these mixtures were separated into 16 components by two-dimensional TLC (Fig. 1B), and furthermore a clear

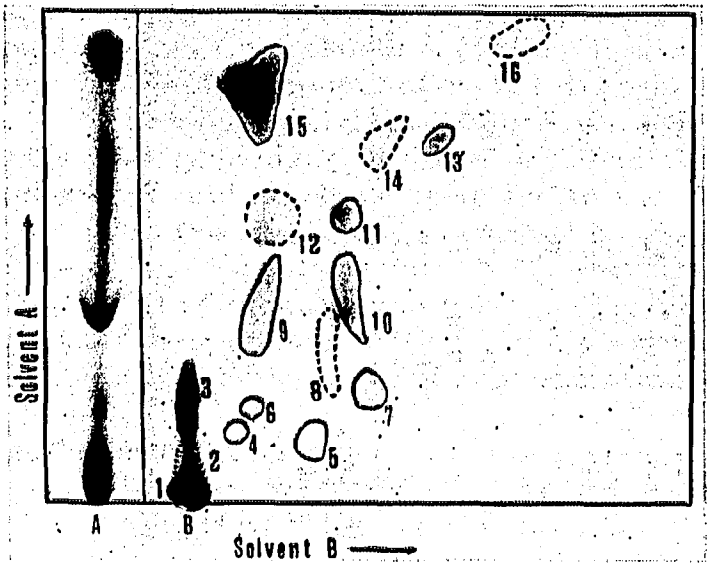

Fig. 1. An autoradiograph showing the separation of rat brain mitochondrial lipids by means of one-dimensional TLC (A) and two-dimensional TLC (B). Compounds (dotted lines) 8, 12, 14 and 16 were detected with iodine. Numbers refer to those given in Table I.

separation of phosphatidyl inositol and phosphatidic acid was achieved (Fig. 1B, Compounds 11 and 15 respectively).

Since these separations were clear and highly reproducible, the present method could be employed in studies on the turnover of the individual phospholipids in brain particulates (Table I).

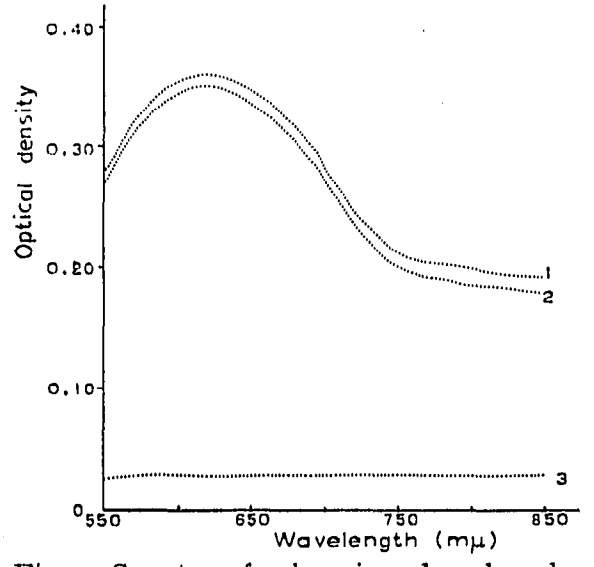

Fig. 2. Spectra of color given by phosphorus assay procedure (see text). (1) 0.2 μmole phosphorus + 100 mg Silica Gel G; (2) 0.2 μmole phosphorus; (3) 100 mg Silica Gel G.

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TABLE I

DETERMINATION OF RADIOACTIVE SPECIFIC ACTIVITIES OF MITOCHONDRIAL AND NERVE-ENDING PHOSPHOLIPIDS AFTER SEPARATION BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

Spot No. (Fig. 1B)	*Compound*	*R_F × 100 in solvents*[a]		*R_F × 100 in solvents*[b]		*Counts/min/spot*		*mμmoles P/spot*		*Specific radioactivity c.p.m./mμmole P*	
		A	*B*	*A*	*B*						
1	Proteolipids + ^{32}P + ganglioside	0	0	0	0	4,460[c]	(900)[d]	14	(—)[e]	319	(—)
2	^{32}P ?	9.2	6.3	—	—	1,974	(400)	0	(0)[f]	—	
3	Unknown	34.8	6.3	—	—	5,370	(2,470)	36	(192)	149	(12.8)
4	Lysolecithin	16.4	14.7	17.8	14.0	67	(29)	71	(4)	0.95	(7.3)
5	Unknown	17.0	28.6	—	—	302	(154)	29.2	(9.6)	10.4	(16)
6	Sphingomyelin	34.1	17.5	31.2	16.0	87	(31)	14	(27)	6.2	(1.2)
7	Unknown	27.8	39	—	—	359		18.7		19.2	
8	Plasmalogen ?	39.2	31.5	—	—	123	(20)	110	(43)	11.2	(0.47)
9	Lecithin	50.0	20.3	48.0	23.0	2,000	(210)	484	(216)	4.2	(0.97)
10	Unknown	50.5	35	—	—	1,974		140		14.1	
11	Phosphatidyl inositol	61.2	36	59.0	33.4	1,026	(2,290)	14	(9)	73.1	(254.3)
12	Phosphatidyl serine	63.2	24.4	65.2	24.0	138	(8)	39	(24)	3.5	(0.33)
13	Unknown	78	54.5	—	—	548		0		—	
14	Phosphatidyl ethanolamine	78	44	77.0	43.1	10	(75)	358	(89)	0.03	(0.84)
15	Phosphatidic acid	90.5	24.5	—	—	7,360	(1,985)	43	(16)	172	(124)
16	Cerebroside	97.5	71.5	96.1	76.2	0	(0)	0	(0)	0	(0)

[a] R_F values of mitochondrial lipids; $R_F = \frac{\text{distance of center of spot from starting-point}}{\text{distance of solvent from starting-point}}$.

[b] R_F values of standard lipids.

[c] Mitochondrial lipids.

[d] Nerve-ending lipids.

[e] Was not determined.

[f] There was no detectable phosphorus in this spot. The radioactivity could have been due to contamination from the ^{32}P-orthophosphate.

As can be seen from Table I, it has not been possible to identify all the lipid components on the thin-layer chromatogram. Compounds 3, 5 and 13 did not stain with iodine but contained a considerable amount of activity. Compounds 3 and 5 could be the deacylated derivative of phosphatidic acid (lysophosphatidic acid) and the phosphorylated derivative of phosphatidyl inositol (diphosphoinositide) respectively. In agreement with the work of other investigators[13,14] phosphatidic acid and phosphatidyl inositol were found to be highly labelled in both nerve-ending particles and mitochondria (Table I). On the other hand structural lipids such as phosphatidyl serine and phosphatidyl ethanolamine had little activity.

Unlike Silica Gel H[10], washed Silica Gel G gave poor resolution of phospholipids. This resulted in higher readings of the Silica Gel G blanks (Fig. 2). However, the blank readings could not have been due to the phosphorus-molybdate complex as can be seen from the silica gel spectrum (Fig. 2). The modification which was made in the developing reagents resulted in a shift of the spectral peak from a maximum at 830 mμ[12] to a maximum at 620 mμ (Fig. 2). The separation and determination of the radioactive specific activities of lipids reported in this communication could be applied to the whole brain as well as particulates from other tissues.

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1 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.
2 W. D. SKIDMORE AND C. ENTENMAN, *J. Lipid Res.*, 3 (1962) 471.
3 S. N. PAYNE, *J. Chromatog.*, 15 (1964) 173.
4 L. G. ABOOD, I. KOYAMA AND V. THOMAS, *Am. J. Physiol.*, 207 (1964) 1435.
5 C. H. REDMAN AND R. W. KENAN, *J Chromatog.*, 15 (1964) 180.
6 A. A. ABDEL-LATIF, *Nature*, 211 (1966) 1530.
7 A. A. ABDEL-LATIF AND L. G. ABOOD, *J. Neurochem.*, 12 (1965) 157.
8 R. P. A. SIMS AND J. A. G. LAROSE, *J. Am. Oil Chemists' Soc.*, 39 (1962) 232.
9 R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45.
10 F. FARKER AND N. F. PETERSON, *J. Lipid Res.*, 6 (1965) 455.
11 E. J. KING, *Biochem. J.*, 26 (1932) 292.
12 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
13 J. GARBUS, H. F. DELUCA, M. E. LOOMANS AND F. M. STRONG, *J. Biol. Chem.*, 238 (1963) 59.
14 J. DURELL AND M. A. SODD, *J. Biol. Chem.*, 239 (1964) 747.

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