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Quantitative Estimation of Some Phosphatides and Their Hydrolysis Products by Thin Layer Chromatography. (28285)

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Many improvements and simplifications have been made in the analysis of phosphatides and water soluble phosphate esters of glycerol. Silicic acid impregnated paper techniques have greatly speeded up the process of identifying and quantitating phosphatides (1,2,3). Dawson and coworkers(4,5) have carried out extensive studies on paper of the hydrolysis products of phosphatides. With the introduction of TLC,* qualitative separation of lipids and phospholipids was speeded up remarkably. The precision of the separations offered the possibility of quantitating the compounds so separated on thin layer plates. Two groups have analyzed some phosphatides quantitatively by TLC(6,7). The separation of the hydrolysis products of phosphatides on TLC has also been reported recently(8,9); however the separations achieved were not suitable for quantitation.

In this report we describe a procedure for quantitative analysis of phosphatides and of the water soluble choline and ethanolamine containing phosphate esters, and apply it to the analysis of serum phosphatides.

Methods. The plates were prepared as previously described(10). Choline chloride and ethanolamine (redistilled) were obtained from Eastman Organic Chemicals, phosphorylcholine (chloride form) and α-glycerophosphate (DL) from Sigma Chemical Co., and o-phosphoethanolamine from California Foundation for Biochemical Research. Lecithin and phosphatidylethanolamine were prepared from egg yolk essentially by the method of Rhodes and Lea(11). Lysolecithin was prepared from egg lecithin as described by Hanahan and coworkers(12). GPC and GPE were prepared from lecithin and PTE respectively according to Dawson(4).

Separation of the choline and ethanolamine derivatives of phosphatides was readily accomplished by the use of 2 solvent systems differing in the amount of acid present. The choline derivatives were separated in a solvent system containing chloroform:methanol: 3M TCA:water, 40:60:20:12.5 (v/v), the solvent run taking approximately 1-1½ hours. The ethanolamine derivatives required less acidic conditions in which the solvent system contained chloroform:methanol:10% TCA:water, 45:55:8:4 (v/v). The phosphatides were separated and identified as previ-

* The following abbreviations are used: TLC, thin layer chromatogram or chromatography; C, choline; PC, phosphorylcholine; GPC, glycerylphosphorylcholine; E, ethanolamine; PE, phosphorylethanolamine; GPE, glycerylphosphorylethanolamine; GP, glycerophosphate; P, phosphate standard; Le, lecithin; LLe, lysolecithin; PTE, phosphatidylethanolamine; Sph, sphingomyelin.

ously described(10) in chloroform:methanol: water, 80:25:3 (v/v).

After chromatography, the plates were dried in an oven at 100° for several minutes. The choline derivatives were identified by the use of 2 sprays. The first spray is a slight modification of the Hanes & Isherwood acid molybdate reagent as described by Dawson (4) consisting of 4 ml 70% perchloric acid, 8 ml 5N HCl, 16 ml 5% ammonium molybdate, 10 ml 3M TCA, and 42 ml of water. The plates were re-dried in the oven after the first spraying. Then followed a light application of the Dragendorf reagent as described by Wagner, Horhammer, and Wolff (13). Choline immediately appeared as a reddish-orange spot whereas the violet blue colored areas of GPC and PC did not appear until several minutes later. GP is identified as a bright blue spot ahead of choline. Ethanolamine derivatives did not stain under the above conditions; however, they were easily identified by application of ninhydrin spray. R_f values obtained with the choline derivatives under above conditions were 0.80, 0.53, 0.32, and 0.20 for GP, C, PC, and GPC respectively; and with the ethanolamine derivatives 0.90, 0.80, 0.40, and 0.18 for GP, E, PE, and GPE respectively.

The ethanolamine derivatives did not interfere with the separation of the choline derivatives in the first solvent system inasmuch as these compounds migrate well ahead of GP near the solvent front. Conversely, the choline derivatives remain at the origin or migrate well below GPE in the second solvent system. Preliminary investigation with several phosphatidase systems has indicated that direct applications of the aqueous reaction mixtures to TLC allows the detection of the above mentioned choline and ethanolamine derivatives. Although the majority of the water soluble contaminants and phosphatides migrated near the solvent front in both solvent systems, these contaminants usually overlapped GP. Thus, it was not always possible to detect GP without further manipulation.

Results. Quantitative procedure. For quantitative estimation, the TLC were separated into lanes 2 cm apart to prevent lateral movement of the solvents. In subsequent studies we have found that with the application of smaller aliquots, lanes 1-1½ cm in width can be used successfully. Samples were applied to each lane with the exception of one lane reserved for controls. After chromatography with the appropriate solvent system and identification with suitable sprays, the spots were delineated into areas approximately 2 cm square and scraped into test tubes. Without further separation of the compounds from the silicic acid(6), phosphorus content was then determined according to the method of Bartlett(14). To each test tube was added 0.16 ml conc. H_2SO_4 followed by digestion in a heating block at 250-260° for 30 min. If less than 0.14 ml of conc. H_2SO_4 is used, the results will be somewhat variable, particularly in the presence of silicic acid. After the tubes were allowed to cool, 1 drop of 30% H_2O_2 was added and the digestion continued for 15 min. With the phosphatides, 3 drops of H_2O_2 were required for complete digestion. 5.0 ml of 0.2% ammonium molybdate and 0.2 ml of Fiske SubbaRow reagent were then added to the cooled solutions, thoroughly mixed, and heated for 10 min. in a steam bath on full force. After cooling, the tubes were centrifuged and the optical density of the clear supernatant was read at 830 $m\mu$ in a Beckman Model B spectrophotometer using water as the reference blank. Under above conditions, 1 μg P (KH_2PO_4) gave an OD reading of 0.164 in absence of silicic acid.

When applied lightly, none of the sprays used for detection was found to interfere with the subsequent phosphorus determination. The presence of molybdate in the acid molybdate spray hastened the digestive process (4); thus, it is possible to further shorten the digestion period. However, heavy application of this reagent to TLC for detection of choline derivatives will result in a bluish background which may give erroneously high phosphorus values. The spots of choline and its derivatives will fade with time but can be made to reappear with a second application of the Dragendorf reagent.

Inhibitory effect of silicic acid. Under some conditions of analysis, silicic acid has not interfered with the color development of phosphorus(2,14); however, under the conditions of our present studies it has. Shen and Dyroff(15) had similarly observed interference by silicates in determination of phosphorus in synthetic detergents.

Of the 8 phosphorus containing compounds tested, the phosphorus color was depressed approximately 14% (range 11-19%) by the silicic acid in an area of 2 sq cm scraped from the TLC (equivalent to about 13 mg of silicic acid), 20% (range 16-22%) by 4 sq cm (about 25 mg of silicic acid), and 25% (range 21-27%) by 6 sq cm (about 38 mg of silicic acid). When 5 different compounds containing from 0.36 to 4.17 μg of P were additionally analyzed in the presence of approximately 25 mg of silicic acid scraped from an area of 4 sq cm on a TLC, the color was depressed approximately 21% (range 17-25%). Thus degree of color suppression is not related to amount of phosphorus added.

Recovery studies. Recovery studies of the individual phosphatides and phosphate esters after chromatography on TLC with suitable solvent systems are given in Table I. Average recoveries of these compounds from mixtures in varying combinations were 102% for Le, 101% for LLe, 95% for PTE, 99% for GPC, and 96% PC. Recovery of these compounds whether done singly or from mixtures was quite reproducible. During these studies, it was noted that approximately 5 to 10% of the applied phosphorus was irreversibly adsorbed at the origin. PE and PTE showed the greatest retention at the origin, approximately 10%. Only 3 to 6% of the phosphorus was retained at the origin in the case of the other compounds. The loss at the origin may be influenced by impurities in the reference compounds. When corrected for this loss (10% for PE and PTE, 5% for all others), quantitative recoveries of the various compounds tested were good, as shown in Table I.

Serum phosphatide values. The serum phosphatides of 7 normal subjects working in this laboratory were also measured quantitatively on TLC. The serum extracts were prepared as described by Vogel, Zieve, and Carleton (2) except that the rotovaped residues were taken up in 1 ml of isoamyl alcohol:benzene (1:1, v/v). 50 μl aliquots were then applied to the TLC and initially chromatographed in petroleum ether:ether:glacial acetic acid, 30:70:1 (v/v) primarily to remove the excess non-phosphatide lipids with the solvent front. After drying, the TLC was rechromatographed in chloroform:methanol:water, 80:25:3 (v/v) and assayed for phosphorus as described earlier.

The phosphorus values of these serum phosphatides from normal subjects are given in Table II. Excellent agreement is revealed in Table III between the average serum phosphatide values obtained with TLC and those obtained with silicic acid impregnated paper chromatograms(2). In terms of percentage of total phosphatide phosphorus measured, these values are quite consistent with the

TABLE I. Recovery of Phosphatides and Water Soluble Phosphate Esters After Chromatography on Silicic Acid Plates.*

Compound	μg P applied	μg P recovered†	% P recovered
Le	1.00	.95	95
	2.00	1.94	97
	3.00	2.91	97
LLe	.70	.68	97
	1.39	1.38	99
	2.09	2.07	99
GPC	1.01	.99	98
	2.03	1.98	98
	3.05	3.04	100
	4.06	3.87	95
PC	.93	.90	97
	1.85	1.74	94
	2.78	2.63	95
PTE	1.24	1.18	95
	2.48	2.41	97
	3.72	3.64	98
GPE	.51	.51	100
	1.03	1.01	98
	1.54	1.44	94
PE	.70	.70	100
	1.41	1.33	94
	2.11	2.03	96

* Avg of 2-4 determinations using 4 sq cm silicic acid.

† Corrected for loss at origin of 10% for PE and PTE, and 5% for other compounds, and corrected for 20% inhibition of phosphorus by silicic acid.

TABLE II. Serum Phosphatide Values Measured In 7 Normal Subjects After Chromatographic Separation on Silicic Acid Plates.*

	μg P per ml				
Subject	Le	LLe	Sph	PTE	TP-P†
1	60.8	9.7	19.1	1.4	91.0
2	70.9	11.6	21.4	2.1	106.0
3	82.3	12.6	28.1	3.2	126.2
4	78.5	13.0	22.9	3.7	118.1
5	68.6	15.3	27.7	4.8	116.4
6	81.8	12.0	28.8	3.2	125.8
7	79.8	12.6	29.2	2.3	123.9
Avg	74.7	12.4	25.3	3.0	115.3

* 4 sq cm areas used.
† Total phosphatide phosphorus.

TABLE III. Comparison of Average Normal Serum Phosphatide Values Obtained by TLC and Impregnated Paper Chromatography.

	μg P/ml		% of TP-P	
Compound	TLC	IPC*	TLC	IPC*
Le	74.7	73.3	64.8	66.4
LLe	12.4	10.5	10.8	9.5
Sph	25.3	24.9	21.9	22.6
PTE	3.0	3.0	2.4	2.7
TP-P	115.3	111.7	99.9	101.2

* Impregnated paper chromatography.

values previously reported by several workers.

GPC determinations run on 2 normal serums and 2 pancreatitis serums did not reveal any GPC in these instances. On the other hand, significant amounts of GPC were present in 2 duodenal drainages aspirated from patients with normal pancreatic function.

Summary. The use of thin layer chromatography for analyzing phosphatides and their hydrolysis products quantitatively is described and applied to the analysis of serum phosphatides. The results are similar to those obtained with silicic acid impregnated paper chromatography and column techniques, though the time for the analysis is markedly shortened.

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Effect of Corticotropin and Growth Hormone on Survival in Hypophysectomized Toads. (28286)

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Toads of the species *Bufo bufo* L. die within a few weeks or months after extirpation of the pars distalis of the hypophysis, length of survival depending mainly upon the temperature. Corticotropin (ACTH) and growth hormone (GH) have both been found very potent in increasing length of survival in *B. arenarum* with extirpated pars distalis (1). We have compared the potency of ACTH and GH in prolonging life in *B. bufo* with extirpated pars distalis.

Methods. Two series of experiments were performed: in the first series, started in May, only females weighing 60-140 g were used; in the second series, started in September-October, equal numbers of females, 50-70 g,