Purification of liver monophosphoinositide

GRAY[1] has recently suggested that many of the solvent systems used in the counter-current distribution of phospholipid mixtures (see [2] for review) could be used on cellulose acetate partition columns to improve these separations. We have used this suggestion in an attempt to simplify the method of MCKIBBIN[3] for the purification of liver monophosphoinositide from contaminating nitrogenous lipids. Hydrolysis with 6 *N* HCl followed by paper chromatography[4] has shown these impurities to be mainly phosphatidyl serine and phosphatidyl ethanolamine.

The inositol-rich lipid fraction was separated from most of the contaminating nitrogenous phospholipids by precipitation from alcohol-chloroform mixtures, in which the latter are more soluble. 300 g crude pig liver lipid (Viobin Laboratories, Monticello, Illinois), which seemed to be essentially similar to the horse liver lipid used by MCKIBBIN[3], were dissolved in 1.4 l chloroform and precipitated with 5 l ethanol. The residue was taken up in 1 l chloroform, filtered through Kieselguhr, and the filtrate treated with 2 l ethanol. The precipitate so formed was further purified by precipitation from 500 ml chloroform with 1.3 l acetone. Five further purifications by the addition in each case of 1.2 l ethanol to 300 ml chloroform were carried out. The non-lipid contaminants were removed by passing the product down a cellulose column[5] in chloroform saturated with water. When all the phospholipid had been washed through, the total volume of eluant was 600 ml. The inositide was precipitated with 940 ml ethanol, allowed to stand for 3 h at 2° C, and finally separated by centrifugation. The product had a N:P molar ratio of 0.33 compared with 1.00 (ref. [2]) and 0.54 (ref. [6]) for previously isolated fractions.

The partition column was prepared by grinding 70 g cellulose acetate (52.5–53.5 % acetyl value, grade CR1FS, 50 mesh from British Celanese Ltd., Hanover Square, London W.1.) with 175–185 ml of the polar phase of the solvent system 280 ml benzene and 120 ml 40°–60° petroleum ether *versus* 240 ml methanol and 80 ml water. This system was based on that used by MCKIBBIN[3] for counter-current distribution, but acetone was omitted from the polar phase. The moist powder was slurried with the non-polar (moving) phase, and made into a column 36 mm diameter. The non-polar phase was passed through until excess stationary phase had been removed. The equilibrium was not readily disturbed by minor changes in temperature. The lipid, prepared as above, was applied in the minimum volume of moving phase and was found to be rapidly partitioned, the purified inositide being eluted with about 250 ml moving phase.

Table I shows that about 70 % of the phospholipid could be recovered, and that although the N:P molar ratios of the products were not constant, between a third and three-quarters of the contaminating lipid nitrogen was removed. These differences do not seem to be accounted for by variations in column "loading". The results show that when the chromatography of the inositide was repeated, the N:P molar ratio was still further reduced, whilst giving good overall phospholipid recovery. We have been unable to purify brain diphosphoinositide preparations using this cellulose acetate partition column technique.

TABLE I

INOSITIDE PURIFICATION ON CELLULOSE ACETATE

	Starting material		*Purified product*	
Source	*N:P molar ratio**	*mg P applied to column*	*N:P molar ratio**	*% recovery of P*
1. Alcohol ppt.	0.33	10.20	0.21	72
2. Alcohol ppt.	0.33	7.14	0.18	66
3. Product from 2	0.18	1.72	0.09	66
4. Alcohol ppt.	0.33	10.20	0.22	67
5. Product from 4	0.22	5.37	0.15	78
6. Alcohol ppt.	0.33	5.10	0.17	67
7. Alcohol ppt.	0.33	5.10	0.14	72
8. Silica fraction (See Table II)	0.18	10.63	0.04	68

The lipid remaining in the stationary phase could be eluted with methanol. It gave N:P ratios of 0.45 and 0.80 respectively in 1 and 8 above. The cellulose acetate is slightly soluble in methanol making recovery of the stationary phase lipid impracticable.

* Nitrogen was determined by the micro-Kjeldahl method, and phosphorus by the method of KING[7].

Using a similar solvent system for counter-current distribution of the lipid mixture, the inositide was found to be concentrated in the polar phase[3]. We expected therefore that the

stationary polar phase, though supported by cellulose acetate, would still preferentially retain the inositide. The reason for the occurrence of the opposite behaviour is not understood. The purification is not due to simple adsorption on the cellulose acetate, since when columns were prepared without stationary phase, none of the applied lipid could be eluted with the moving phase.

To compare the behaviour on silicic acid of our inositide preparation (N : P molar ratio 0.33) with those of McKIBBIN[3], we followed his method of elution using increasing concentrations of methanol in chloroform. The fractionation produced was found to vary greatly with the adsorptive capacity of the silicic acid used. Untreated Mallinckrodt "acid silicic" (100 mesh, "Suitable for chromatographic analysis by the method of Ramsey and Patterson") adsorbed the applied lipid so strongly that an inositide-rich fraction could only be eluted with pure methanol, giving poor fractionation. When this silicic acid was washed with distilled water to remove fine particles, dried with acetone, and heated overnight at 110° C, the results (Table II) showed an improved separation, especially in the fraction eluted by 17.0 % methanol in chloroform. Finally "Precipitated Silica" (British Drug Houses Ltd., Poole, England) was used. This was similarly washed with water, then with *N* HCl, and again with water until the pH of the supernatant was about 5.5, dried with acetone, and heated overnight at 110° C. This enabled a greatly purified inositide to be obtained from the 10.5 % and 17.0 % methanol in chloroform fractions, though the phospholipid recovery was only 16.2 %.

TABLE II

INOSITIDE PURIFICATION ON SILICIC ACID COLUMNS

Silica used	*Starting material N : P molar ratio*	*Eluant: % MeOH in $CHCl_3$*	*% recovery of P applied*	*Purified product N : P molar ratio*
Mallinckrodt washed	0.33	6.0	1.88	
		10.5	5.03	
		17.0 (1st 400 ml)	10.97	0.18*
		17.0 (2nd 400 ml)	6.75	0.21**
		30.0	12.31	0.10
		100.0	21.36	0.14
B.D.H. washed	0.33	6.0	25.9	0.5***
		10.5 plus 17.0	16.2	0.11
		100.0	23.1	0.7***
McKIBBIN[3]	1.00	2.0	0	Not stated
		6.0	Not stated	Not stated
		9.0	Not stated	Not stated
		17.0	92.7	0.79
McKIBBIN[3]	0.10 (a)	13.0	approx. 1.0	0.00 (a)
	0.15 (b)	13.0	approx. 1.0	0.08 (b)

* Used as starting material on cellulose acetate (Table I).

** Precipitate came out on standing, and had a N : P molar ratio of 0.07. Acid hydrolysis showed inositol and glycerol present.

*** Approximate values by chromatography of hydrolysates.

The results suggest that silicic acid, like alumina, varies greatly in adsorptive power, and that in this separation a weakly adsorbing silica is required. All these silicic acid columns, however, gave low recoveries of purified inositide, and were often found to have a slow rate of solvent flow even when mixed with celite. The cellulose acetate columns seem preferable for these stages of purification, since they have faster rates of solvent flow and give higher yields. They are readily adapted to give successive purifications using two or more columns.

We are indebted to the Medical Research Council for a grant to one of us (R. R. D.).

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Received May 6th, 1957