Chromatography of acidic phospholipids on immobilized neomycin

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Summary Columns of immobilized neomycin (reductively coupled to porous glass beads) were used to separate weakly acidic lipids as well as the polyphosphoinositides. All anionic lipids present in chloroform–methanol extracts, which had been washed first with acid and then with neutral salt solutions, were adsorbed. Phosphatidylserine and phosphatidic acid were eluted with chloroform–methanol–formic acid mixtures. Phosphatidylinositol and cardiolipin were eluted sequentially by very low concentrations (10–100 mM) of ammonium formate in chloroform–methanol–water. All three phosphoinositides were isolated from washed chloroform–methanol–KCl extracts of brain. Sulfatides eluted with the phosphatidylinositol. Phosphatidylinositol phosphate and phosphatidylinositol bisphosphate were eluted in turn by higher salt concentrations (0.4–1 M). The immobilized neomycin was used repeatedly but the capacity eventually declined. This loss was reversed by sodium borohydride reduction.—**Palmer, F. B. St. C.** Chromatography of acidic phospholipids on immobilized neomycin. *J. Lipid Res.* 1981. **22:** 1296–1300.

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The antibiotic neomycin exhibits a specific affinity for PIP and PIP_2. Presumably this results from a strong ionic interaction between the cluster of six primary amino groups on the neomycin and the several negatively charged phosphate groups on the lipid molecules. There is no great affinity for other anionic phospholipids, including PI. This specific interaction is the basis for the chromatographic isolation of these polyphosphoinositides using columns of porous glass beads to which neomycin has been covalently bonded (1). Neomycin can also act as a weak anion exchanger and therefore offers some potential for the separation of other anionic phospholipids. The neomycin-glass adduct exhibits many desirable features. It has a high capacity, exhibits a high flow rate with organic solvents, is stable in such solvents in the presence of salts, acids, and bases, and can be reused repeatedly (1). Therefore, the anion exchange properties of immobilized neomycin were explored as a preparative method for the rapid isolation of the other less acidic phospholipids and for the single step analysis of all three phosphoinositides in lipid extracts of brain.

Abbreviations: PA, 3-*sn*-phosphatidic acid; P_2G, 1,3-bis(3-*sn*-phosphatidyl)glycerol; PS, (3-*sn*-phosphatidyl)-L-serine; PI, 1-(3-*sn*-phosphatidyl)-D-myo-inositol; PIP, 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4-phosphate; PIP_2, 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4,5-bis(phosphate); SU, sulfatide; TLC, thin-layer chromatography.

METHODS

All solvents were saturated with N_2. The proportions of solvent mixtures are expressed by volume. Rats (Sprague-Dawley, 250–350 g) were killed by decapitation. The brain or liver was homogenized in 10 vol of chloroform–methanol 1:1 with a Polytron (Brinkman Instruments) and the tissue residue was re-extracted with 10 vol of chloroform–methanol 2:1. The combined extracts were diluted with chloroform to raise the chloroform–methanol ratio to 2:1 and were shaken with 0.2 volume of 1 N HCl. The resulting lower phase was washed twice with an equal volume of methanol–0.1 M KCl 1:1. The organic solvents were removed under vacuum and the lipids were dissolved in 5–10 ml of chloroform–methanol 1:1. Extracts of brain containing the polyphosphoinositides were obtained using the biphasic extraction system containing KCl (1). The chloroform-rich lower phases were washed once with an equal volume of methanol–1 M HCl 1:1 and then twice with methanol–0.5 M KCl 1:1. The washed chloroform layer was mixed with an equal volume of methanol just prior to column chromatography.

Neomycin sulfate (Sigma Chemical Co.) was reductively coupled to reactive porous glass beads (Glycophase G/CPG-200, 200/400 mesh; Pierce Chemical Co., Rockford, IL) as described by Schacht (1). The neomycin-coated glass beads were packed into small columns, acid-stripped, converted to the required salt form, and equilibrated with the starting solvent by eluting in order with 3 column volumes each of chloroform–methanol–3 M HCl 5:10:2, chloroform–methanol–water 5:10:2, 0.5 M ammonium formate or acetate in chloroform–methanol–water 5:10:2, chloroform–methanol–water 5:10:2, and finally chloroform–methanol 1:1. After removal of any insoluble material by centrifugation, the lipid extract (in 5–7 column volumes of solvent) was passed through the column followed by 3 column volumes each of chloroform–methanol 1:1 and chloroform–methanol 1:2. The adsorbed anionic lipids were eluted as described below. Eluates containing acetic or formic acid in chloroform–methanol 1:2 were washed first by adding 5 ml water per 10-ml eluate. Eluates containing ammonium salts in chloroform–methanol–water 5:10:2 were washed by adding 3 ml of HCl solution per 10-ml eluate (1 M, 3 M, and 6 M

HCl for eluates containing 0–0.1 M, 0.15–0.5 M, and 0.6–1.0 M salt, respectively). The resulting lower phases were washed once with an equal volume of methanol–1 M HCl 1:1 and with methanol—0.5 M KCl 1:1. Washed fractions were dried under N_2 and stored in chloroform at −20°C.

Lipids were separated on pre-coated silica gel HR plates (Analtech Inc., Newark, NJ) developed with chloroform–methanol–acetic acid–water 100:45:20:7 or chloroform–ethanol–triethylamine–water 30:35:35:8 (2, 3). The plates were used as supplied without further activation. The pre-coated plates were also dipped in 1% (w/v) potassium oxalate, dried, and reactivated by heating at 120°C for 40 min. These chromatograms, developed with chloroform–acetone–methanol–acetic acid–water 40:15:13:12:8, were used mainly for the separation of polyphosphoinositides (4). Phospholipids were located with the molybdate spray reagent (5) or by charring with 3% cupric acetate in 8% phosphoric acid (6). Identification of phospholipids in column eluates was based on a comparison of their chromatographic mobilities with those of marker phospholipids (Serdary Research Laboratories, London, Canada) in all three thin-layer chromatographic systems. Lipid phosphorus was determined by the method of Bartlett (7) after digestion of the lipids with perchloric acid.

RESULTS

Anionic phospholipids (18–22% of the total lipid phosphorus) were adsorbed by the immobilized neomycin from acid-washed and salt-washed lipid extracts of both liver and brain. Up to half of the PS and PI was not adsorbed if the acid washing step was omitted, presumably due to the presence of calcium and magnesium salts of these lipids. The final neutral salt wash of the extracts was also necessary since any residual traces of HCl displaced the weakly bound PS and PI. Neutral lipids, zwitterionic phospholipids, and their lyso derivatives were not adsorbed. They were removed from the column with chloroform–methanol 1:1 and 1:2. The preferred elution scheme is summarized in **Table 1.** A brown pigment, present in larger quantities in liver extracts, was adsorbed. This material was displaced by the anionic lipids and moved down the column as a discrete band during application of the extract. Its location was used as an index of column saturation and was located one-half to three-quarters of the way down the column in most experiments. This pigment was selectively removed in 2–3 column volumes by adding a very small amount of formic acid to the eluting solvent (chloroform–methanol–88% formic acid 300:600:1). Over 90% of the PS was eluted next in 6 column volumes of chloroform–methanol–88% formic acid 10:20:1 (see **Fig. 1**). This lipid was quite pure (Fig. 2), however, the trailing edge of the peak eluted in the next 6 column volumes of the same solvent was contaminated with PA. Elution with (6 column volumes) chloroform–methanol–88% formic acid 5:10:1 ensured removal of this lipid. When PA was added to tissue extracts most of it was eluted by this solvent but some also appeared in the tail of the PS peak. Prolonged elution with this solvent also resulted in leakage of the PI. Flushing the acid from the column with 2 column volumes of chloroform–methanol–water 5:10:2 reduced the tendency of P_2G (cardiolipin) to elute prematurely with the PI which was removed next by 6 column volumes of 20 mM ammonium formate in chloroform–methanol–water 5:10:2. This fraction from

TABLE 1. Elution of immobilized neomycin columns

Step	Solvents[a]	Elution Volumes[b]	Lipids Eluted
1	C–M 1:1	2–4	all nonacidic
2	C–M 1:2	2–4	lipids
3	C–M–F 300:600:1	2–3	pigments
4	C–M–F 10:20:1	6	PS
5	C–M–F 10:20:1	6	PS, some PA
6	C–M–F 5:10:1	6	PA
7	C–M–H_2O 5:10:2	2	none
8	20 (40) mM ammonium formate[c]	6	PI, sulfatide (brain)
9	100 (200) mM ammonium formate[c]	6	P_2G
10	400 (600) mM ammonium formate[c]	6	PIP
11	1 M ammonium formate[c]	8–12	PIP_2

[a] Abbreviations for solvents: C, chloroform; M, methanol; F, 88% formic acid.
[b] Elution volumes are specified as column volumes, volume occupied by the column packing.
[c] Ammonium formate was dissolved in C–M–H_2O 5:10:2. The higher concentrations given in brackets were used with the second batch of neomycin beads.

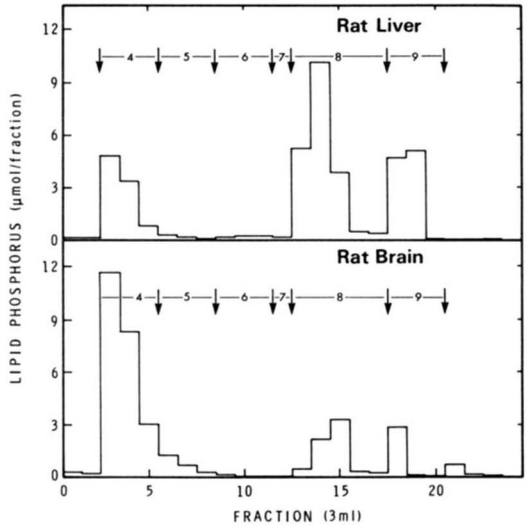

Fig. 1. Fractionation of acidic phospholipids on immobilized neomycin. Chloroform–methanol extracts of rat liver (6.2 g) and rat brain (3.8 g) prepared as described and containing 220 and 200 μmol lipid phosphorus, respectively, were applied to 1 × 2-cm columns in chloroform–methanol 1:1. The columns were flushed with 3 column volumes of chloroform–methanol 1:1 and 1:2 and then eluted step-wise, each fraction being 2 column volumes of solvent. Solvents were changed at the fractions indicated. Numbers between the arrows correspond to the steps in the elution scheme summarized in Table 1. Stripping of any remaining lipid from the column with chloroform–methanol–3 M HCl 5:10:2 was begun after step 9.

liver extracts contained a single ninhydrin negative, phosphorus positive spot on TLC (**Fig. 2**). However, the fraction from brain extracts contained a second phosphorus-negative material which was identified as sulfatide based on its reaction with orcinol and on co-chromatography with authentic bovine brain sulfatide (Sigma) in both the acidic and basic TLC systems. Continued elution with 20 mM ammonium formate removed a very small additional amount of PI which was contaminated with the next more tightly bound lipid. This lipid was eluted with 2–4 column volumes of 100 mM ammonium formate in chloroform–methanol–water 5:10:2 and consisted of one major phospholipid identified as P_2G (Fig. 2). However, charring the chromatogram revealed the presence of small quantities of other lipids which were not visualized by the molybdate spray for phosphorus.

The separation was not affected by flow rate. Columns were run unrestricted with a head pressure of 2–3 column volumes of solvent resulting in flow rates of 0.3–0.5 column volumes per min. The capacity of the neomycin beads was high. Although about 150 μmol lipid phosphorus/ml of beads was used in most studies, comparable separations have been obtained with loads in excess of 230 μmol/ml. This represents a binding capacity for these weakly acidic phospholipids of at least 40 μmol/ml, a value considerably higher than that achieved with polyphosphoinositides (1).

Similar results were obtained with an acetic acid–ammonium acetate elution scheme. PS was eluted more slowly by acetic acid-containing solvents, 90% in 15 and 10 column volumes of chloroform–methanol–acetic acid 5:10:2 and 5:10:3, respectively. Very prolonged elution (50 column volumes) was necessary to remove all the PS and PA present in brain extracts. PI and P_2G could then be eluted with 10 mM and 50 mM ammonium acetate in chloroform–methanol–water 5:10:2, respectively. The amounts of acidic phospholipids recovered from brain and liver (**Table 2**) are similar to values reported in the literature. Our value for PS may be slightly high due to inclusion of the small amount of PA which elutes in the trailing edge of the PS peak.

As reported by Schacht (1), the neomycin-glass beads could be reused repeatedly if carefully stripped and re-equilibrated between samples. However, the chromatographic capacity did decline with use and the beads became discolored. The original properties could be restored by repeating the last step of the immobilization procedure, the sodium borohydride reduction. The separations shown in Fig. 1 were achieved with a batch of neomycin beads which was a year old and had been re-reduced three times.

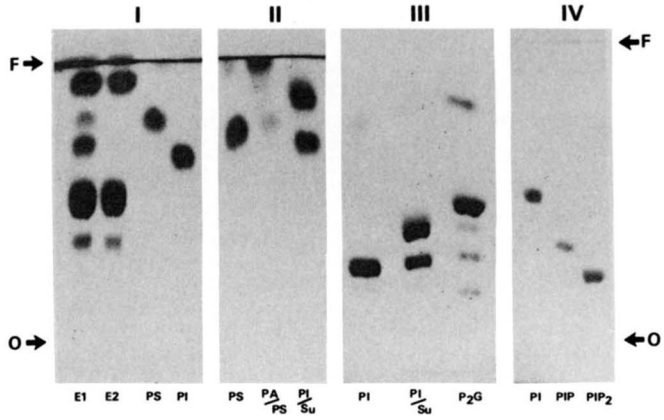

Fig. 2. Thin-layer chromatography of fractions from immobilized neomycin columns. Chromatograms 1 and 2 were developed in the acidic solvent. Chromatogram 1 shows the total lipid extract from liver before (E1) and after (E2) passage through the column, as well as the PS and PI obtained in steps 5 and 8 of the elution scheme (Table 1). Chromatogram 2 shows the PS, PA/PS, and the PI/Su obtained from extracts of brain in steps 4, 5, and 8, respectively. The same liver PI and brain PI/Su in addition to liver P_2G (step 9) is shown in chromatogram 3 when developed in the alkaline solvent. Lipids were detected by charring in chromatograms 1–3. Chromatogram 4 shows the PI, PIP, and PIP_2 isolated from chloroform–methanol–KCl extracts of brain (steps 8, 10, and 11) when chromatographed on oxalate-treated plates and located with the phosphorus-detecting reagent.

TABLE 2. Recovery of acidic phospholipids by chromatography on immobilized neomycin

	C–M Extract		C–M–KCl Extract
	Liver (n = 7)	Brain (n = 3)	Brain (n = 5)
	μmol/g wet weight[a]		
Total phospholipid	35.9 ± 2.7	54.2 ± 4.8	54.1 ± 0.7
PS	1.38 ± 0.18	7.84 ± 0.64	
PI	3.32 ± 0.31	1.79 ± 0.13	1.74 ± 0.08
P_2G	0.84 ± 0.11	0.50 ± 0.20	0.39 ± 0.09
PIP			0.25 ± 0.02
PIP_2			0.38 ± 0.01

[a] All values are the mean ± SD.

Although prepared according to the same protocol, a second batch of neomycin beads bound the acidic phospholipids more strongly than the first. In this case PS elution was slightly delayed and 40 mM and 200 mM ammonium formate in chloroform– methanol–water 5:10:2 were required to elute PI and P_2G, respectively.

Small columns of immobilized neomycin were also used to isolate all three inositol phospholipids from the chloroform–methanol–KCl extract of a rat brain in a single column run lasting only 2 hr. The acid- and salt-washed extract from one brain, containing about 100 μmol of phospholipid, was passed through a 0.7 × 2 cm column of neomycin beads. Separate recovery of the pigment, PS, and PA was not desired. Therefore, these lipids were removed from the column with an abbreviated elution scheme in which steps 3 and 5 (Table 1) were omitted. After removing acid from the column with chloroform–methanol–water the PI/sulfatide, P_2G, and PIP were eluted sequentially with 6 column volumes each of 20 mM, 100 mM, and 400 mM ammonium formate in chloroform–methanol–water 5:10:2, respectively. PIP_2 was eluted with 8 column volumes of 1 M ammonium formate in chloroform–methanol–water 5:10:2. Separations using the second batch of immobilized neomycin were achieved by increasing the concentration of salt to 40 mM, 200 mM, and 600 mM for PI, P_2G, and PIP, respectively, and prolonging the elution with 1 M ammonium formate to 12 column volumes for PIP_2. TLC (Fig. 2) revealed only one phosphorus-staining band for each of the inositol phospholipids. Difficulty in eluting the columns with 1 M salt reported earlier by Schacht (1) for ammonium acetate was not encountered with ammonium formate and PIP_2 prepared this way did not exhibit the slower moving contaminant which was eluted from the columns by strongly alkaline or acidic solvents (1).

The neomycin columns were more sensitive to loading when chloroform–methanol–KCl extracts were used. The recovery of PI declined when the load exceeded 150 μmol phospholipid phosphorus/ml of beads. Even at the lower loads (110–130 μmol/ml) used above, only half of the PS was adsorbed onto the column. However, 0.75 ml of beads was sufficient for the complete recovery of all three phosphoinositides from one rat brain weighing 1.5–1.8 g. Some PIP and PIP_2 was lost during removal of ammonium formate from the eluted lipids by washing in the biphasic system. When an accurate estimate of the phosphoinositide content of the tissue was required (Table 2), aliquots of the eluted fractions were digested for phosphorus analysis without prior removal of the salt. The values compare very favorably with those reported for all three phosphoinositides in rat and chicken brain extracted and analyzed by different methods (2, 8) and with guinea pig brain polyphosphoinositides isolated on neomycin columns (1).

DISCUSSION

This study extends the use of immobilized neomycin to include the isolation of anionic lipids other than the polyphosphoinositides. Conditions for the elution of PS, PA, PI, P_2G, and sulfatide are similar to those used with DEAE and TEAE celluloses (9). The porous glass beads provide a stable, rigid matrix, which permits reuse of the columns with only infrequent repacking. The neomycin adduct was stable in the solvent mixtures used but, when performance did decline after extensive use, the original chromatographic characteristics could be restored by reduction with sodium borohydride. The capacity of the neomycin beads was high and pure fractions were obtained with stepwise elution. As noted before (1), a better separation of PIP and PIP_2 could be obtained than is possible with DEAE cellulose. The formate elution scheme offers several advantages over the acetate system: PS and PA are more rapidly eluted by solvents contain-

ing formic acid, and PIP_2 is completely eluted by 1 M ammonium formate without the additional contaminant that is eluted by solvents containing HCl or ammonia (1).

Pure PS was obtained from both liver and brain extracts when the trailing edge of the peak was discarded to avoid some contamination with PA. The PI isolated from liver was quite pure; however, the sulfatide in extracts of brain was eluted with the PI. For preparative purposes sulfatide, PA, and P_2G could be removed from the extracts by prior chromatography of the extracts on silicic acid (10). Silicic acid could also be used to remove these contaminants from the neomycin eluates, as would be necessary with brain samples from which the polyphosphoinositides were also to be isolated.

In the case of brain, it is desirable to isolate all three phosphoinositides from a single extract by one rapid column chromatographic step. The polyphosphoinositides represent a very small proportion of the phospholipids in all tissues. To facilitate their isolation they are usually extracted separately from other phospholipids. There is a potential for loss in some of the dual extraction procedures used for this purpose (2, 8). In these procedures, as well as in the more recent methods in which immobilized neomycin was used only to isolate PIP and PIP_2 (11, 12) from a single extract, it is still necessary to recover PI from the remaining lipids. Separations of this type are made difficult by the small proportion of PI relative to that of PS in extracts of brain and usually require two-dimensional chromatography. The methods described here avoid these difficulties and provide for the analytical and preparative isolation of all three phosphoinositides from a single extract in one rapid chromatographic step. The presence of sulfatide in the PI does not interfere with quantitation based on phosphorus analysis nor with metabolic studies using ^{32}P. Radioactive myo-inositol was also found not to be incorporated into sulfatide. If necessary, the sulfatide may be removed from the PI on silicic acid or by one-dimensional TLC.

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