notes on methodology

Purification of lipids from nonlipid contaminants on Sephadex bead columns

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SUMMARY A simple, rapid column procedure for the removal of nonlipid contaminants from lipid extracts is described. Lipid P is completely recovered and nonlipids completely removed.

KEY WORDS lipids · purification · nonlipids · cartilage · column · Sephadex

Separation of lipids from nonlipid contaminants in chloroform–methanol extracts of tissues has most commonly been accomplished by the washing procedures of Folch and coworkers (1, 2). In our experience with lipid extracts from cartilage and bone, however, very stable emulsions were formed which could not be broken even by repeated freezing and thawing. These cartilage extracts contained large amounts of nonlipid material. Cellulose columns (3) and more recently Sephadex G-25 columns (4) were tested for their efficacy in removing nonlipids. Although the cellulose columns were simple and rapid to prepare and use, we have confirmed the finding of Garcia, Lovern, and Olley (5) that phosphatidyl inositol was not quantitatively recovered. On the other hand, difficulty was experienced with the Sephadex method, which failed to remove all of the nonlipids. Failure was probably caused by not strictly adhering to the very slow flow rate specified by the authors. Similar difficulty with this method was experienced by Siakotos and Rouser (6), who recently reported a Sephadex procedure which permits separation of water-soluble nonlipids, gangliosides, and other lipids. Another Sephadex method for separation of steroids from nonsteroids in urine has been reported by Gupta (7). However, since the conditions required did not seem applicable to phospholipids, this procedure was not tested.

Reported here is a new procedure utilizing bead-form G-25 Sephadex which affords equal or better removal of nonlipids than the method of Wells and Dittmer (4). It permits much faster flow rates and has the added advantage that the columns can be easily regenerated and used again. The procedure has permitted complete recovery of total lipid P, no change being noted in the distribution of P among the individual phospholipids. If desired, the nonlipids present in the crude lipid can also be conveniently recovered. Its main advantage over the method of Siakotos and Rouser (6) is that it is simpler.

Preparation of Solvents. Redistilled reagent grade chloroform and methanol were used to prepare the solvents. The upper and lower phases formed by the mixture chloroform–methanol–water (200:100:75, by volume) were collected separately and stored at room temperature until used; they are referred to hereafter as UP and LP, respectively.

Sephadex Column Preparation. Chromatography columns (1 cm I.D. × 15 cm) equipped with sintered glass bottoms were used. Sephadex G-25 (fine, bead-form, Pharmacia Fine Chemicals, Piscataway, New Market, N.J.), 25 g, was soaked overnight in 100 ml of UP, then rinsed with 4 × 100 ml of UP. The columns were packed by pipetting a sufficient volume of the slurry of swollen Sephadex beads into the chromatography tubes to form 10-cm high columns when settled. Bubbles were prevented from being trapped in the columns by pipetting the slurry into a small volume of UP previously added to the chromatography tubes. The Sephadex was allowed to settle under slight nitrogen pressure (1–2 psi) and the columns were capped with filter paper disks. The columns were then rinsed with 10 ml of UP, followed by 10 ml of LP before addition of the crude lipids. The void volume of the columns was 2.3 ml.

Preparation of Lipid. Crude lipid extracts of tissues obtained by the procedure of Folch, Lees, and Sloane Stanley (2) were concentrated to small volume under reduced pressure in an atmosphere of nitrogen and finally taken to complete dryness under high vacuum. Similarly, crude lipid extracts obtained from preextracted tissues by the acidic chloroform–methanol extraction method of Folch (8) were neutralized with dry $NaHCO_3$ or ammonia vapor, filtered, concentrated, and dried as described above.

Sephadex Chromatography. The dry crude lipid (up to 200 mg) was taken up with successive small volumes (2–5 ml) of LP, which were transferred to a medium porosity sintered glass funnel (to remove precipitated proteins) and filtered directly onto the columns. Purified lipid was then eluted from the columns with sufficient additional LP to make a total of 25–30 ml of effluent (fraction 1). The flow rate (up to 1 ml/min) was controlled by nitrogen pressure (2–3 psi) on the column. Pressure in excess of 5 psi caused excessive "bleeding"

Abbreviations: UP, upper phase of Folch system; LP, lower phase. Solvent ratios are v/v.

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of UP. The small volume of UP (ca. 0.5 ml) which ordinarily bled from the column upon elution of the lipid could conveniently be removed by passing the eluate through dry filter paper. However, since some lipid could be lost in this procedure and since the aqueous phase contained no appreciable nonlipid material, this step was usually omitted.

Drying of Purified Lipid. Because the LP contained significant amounts of water, care was taken in drying the purified lipid to prevent possible lipid hydrolysis. The eluate was concentrated under a reduced pressure of nitrogen at room temperature to a small volume, whereupon a cloudy aqueous emulsion formed. This was frozen in the flask over solid CO_2 and lyophilized. The dried lipid was taken up in a small volume of chloroform–methanol, transferred to tared vials, evaporated in a stream of nitrogen, dried under high vacuum over paraffin shavings, and weighed.

Recovery of Lipid Phosphorus. In studies of the recovery of lipid P, crude extracts of calf heart, liver, or cartilage in LP were used. Aliquots of the crude lipid solution were taken for analysis of total P (heart and liver), for paper chromatographic analysis (9) of the distribution of P among the individual phospholipids (heart and cartilage), and for chromatography on Sephadex columns. The purified lipid was collected, taken to dryness as previously described, and made to known volume. Samples were taken for analysis of total P (heart and liver) and for paper chromatographic analysis of the individual phospholipids (heart and cartilage). In additional experiments aliquots of purified lipid in LP were taken for rechromatography on Sephadex and for total P analysis. The repurified lipid was collected from the columns, taken to dryness, and made to known volume. Samples were taken for analysis of total P.

Tests for Removal of Nonlipids. A solution of crude calf liver lipids in chloroform–methanol 2:1 was used. Solutions of carrier-free inorganic phosphate-^{32}P, uniformly labeled glucose-^{14}C, glycine-2-^{14}C, or unlabeled adenosine triphosphate (disodium salt) were added to aliquots of the crude lipid solution in a round bottom flask. The flask contents were evaporated to dryness under a reduced pressure of nitrogen. The crude lipids (with added nonlipid) were taken up with LP and made to known volume. Equivalent samples were taken for chromatography on Sephadex columns and for analysis of total nonlipids (column load). The loads of crude lipids ranged from 50 to 100 mg/column. The purified lipid was eluted from the columns with 25 ml of LP (fraction 1). Subsequently, 50 ml of UP was added to each column for elution of the nonlipid (fraction 2). This was followed by an additional 50 ml rinse of UP (fraction 3), as a check on complete recovery of the nonlipid. The three eluates from the column were made to known volume and suitable samples taken for analysis of nonlipids.

Regeneration of Columns. The Sephadex columns were regenerated after each use by washing with at least 50 ml of UP to remove nonlipids. This was followed by rinsing with 20 ml of LP to prepare the column for the next lipid sample. After repeated regeneration, flow rates decreased to the point where repacking of the columns was advisable.

Chemical and Radioactivity Determinations. Total phosphorus determinations were by a modification (9) of the Martin and Doty (10) method. Adenosine triphosphate was determined from the absorbancy at 259 mμ, correction being made for the absorbancy of the pure lipid. Radioactivity measurements were made using a Nuclear-Chicago automatic gas-flow counter. Aliquots were dried on 3-cm planchets and counted to predetermined total counts. Appropriate corrections for sample thickness and background were made.

Lipid Phosphorus Recovery. Data presented in Table 1 demonstrate that the total P load of the columns was recovered in the lipid fraction, whether crude or previously purified lipid was applied to the column. It seems, therefore, that only traces of nonlipid P were present even in the crude extracts. Complete recovery of lipid P was further supported by the results presented in Table 2, which indicate no significant difference in the distribution of lipid P among the various phospholipids of calf heart and cartilage after chromatography on Sephadex. Sephadex, in contrast to cellulose, seems to bind none of the individual phospholipids preferentially.

Removal of Nonlipids. Table 3 illustrates that when crude calf liver lipids mixed with nonlipids were chromatographed on Sephadex columns, in all cases less than 1% of the added nonlipid was recovered in the lipid (fraction 1). When removal of traces of radioactive nonlipids was tested, no more than 0.15% and as little as 0.04% of the total radioactivity contaminated the lipid. The data further demonstrate that 99% or more of these nonlipids can be recovered from the column by rinsing with 50 ml of UP (fraction 2). An additional rinsing with 50 ml of UP (fraction 3) removed less than 1% of the total nonlipid material. Within the limits of accuracy of the experimental methods, all of the added nonlipid was accounted for in the three fractions. The variations in the apparent recovery of the column load can be explained by the fact that the nonlipids did not form truly homogeneous solutions with the crude lipid. As a result, variations occurred both in the true column load and in the aliquots taken for direct analysis and not put through the column. When the data were expressed as a percentage of the recovered nonlipid from each column (Table 3), more representative and consistent results were obtained.

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TABLE 1 RECOVERY OF LIPID PHOSPHORUS OF CRUDE AND PURIFIED LIPIDS FROM SEPHADEX COLUMNS

Lipid Sample	No. of Columns	Column Load	Lipid Fraction	Recovery
		mg total P		%
Calf liver (crude)	4	1.184 ± 0.007	1.184 ± 0.009	100.0 ± 0.8
Calf heart (crude)	2	2.612 ± 0.000	2.588 ± 0.025	99.5 ± 0.5
Calf heart (purified)	4	1.673 ± 0.025	1.678 ± 0.010	100.1 ± 0.6
Rat liver (purified)	2	0.968 ± 0.007	0.962 ± 0.007	99.4 ± 1.3

Values are means ± SEM. Column load equals the total P applied to the columns; lipid fraction is the total P recovered in fraction 1; per cent recovery is the percentage of the column load of P recovered in the lipid fraction.

TABLE 2 RECOVERY OF INDIVIDUAL PHOSPHOLIPIDS FROM CRUDE LIPID EXTRACTS PASSED THROUGH SEPHADEX COLUMNS

Lipid	Calf Heart Lipids		"Residual" Cartilage Lipids*	
	Before Sephadex	After Sephadex	Before Sephadex	After Sephadex
	% of total lipid P			
Nonmigrating material	0.2 ± 0.1	0.2 ± 0.2	5.3 ± 1.6	0.5 ± 0.0
Sphingomyelin	9.8 ± 0.9	9.1 ± 0.3	5.5 ± 0.9	5.0 ± 0.4
Phosphatidyl choline	43.5 ± 0.6	42.9 ± 0.6	48.9 ± 0.5	51.4 ± 1.4
Lysophosphatidyl choline	0.3 ± 0.1	0.2 ± 0.1	7.6 ± 1.5	7.9 ± 0.3
Phosphatidyl ethanolamine	27.5 ± 0.2	27.7 ± 0.3	11.9 ± 1.1	12.8 ± 0.5
Lysophosphatidyl ethanolamine	— —	— —	12.8 ± 0.4	10.9 ± 0.8
Phosphatidyl inositol	3.4 ± 0.2	3.4 ± 0.1	3.4 ± 0.1	2.9 ± 0.1
Phosphatidyl serine	3.4 ± 0.2	3.4 ± 0.1	4.6 ± 0.2	4.4 ± 0.4
Phosphatidic acid	tr.	tr.	— —	— —
Diphosphatidyl glycerol	11.0 ± 0.8	12.1 ± 0.3	†	0.8 ± 0.2
Unidentified	0.4 ± 0.1	0.6 ± 0.1	†	0.6 ± 0.3
"Chondrolipin"‡	— —	— —	1.0 ± 0.1	1.3 ± 0.0

Quadruplicate samples of crude and Sephadex purified calf heart lipids, containing 13.5 and 9.3 μg lipid P respectively, were analyzed by paper chromatography. Duplicate samples of crude and Sephadex-purified "residual" calf cartilage lipids, containing 3.0 and 6.1 μg lipid P respectively, were similarly chromatographed and analyzed. Values represent means ± SEM.

* Extracted with acidified chloroform–methanol.

† Too little P for analysis.

‡ "Chondrolipin" is a new, partially characterized lipid found in cartilage.

TABLE 3 SEPARATION AND RECOVERY OF PHOSPHATE-^{32}P, GLUCOSE-^{14}C, GLYCINE-^{14}C, AND UNLABELED ATP FROM CALF LIVER LIPIDS ON SEPHADEX COLUMNS

Nonlipid Applied	Fraction 1 (Lipid)	Fraction 2 (Nonlipid)	Fraction 3 (Residual)
		% of recovered nonlipid	
Phosphate-^{32}P	0.042 ± 0.015	99.17 ± 0.08	0.79 ± 0.09
Glucose-^{14}C	0.147 ± 0.003	99.82 ± 0.01	0.030 ± 0.015
Glycine-^{14}C	0.097 ± 0.004	99.61 ± 0.24	0.29 ± 0.22
ATP (unlabeled)	0.90 ± 0.07	98.69 ± 0.30	0.41 ± 0.22

Carrier-free inorganic phosphate-^{32}P (2.093 ± 0.069 × 10^4 cpm) applied to three columns, 2.094 ± 0.022 × 10^6 cpm recovered; 4.52 μmoles of uniformly labeled glucose-^{14}C (1.454 ± 0.012 × 10^6 cpm) applied to four columns, 1.472 ± 0.020 × 10^6 cpm recovered; 0.456 μmole of glycine-2-^{14}C (1.283 ± 0.045 × 10^6 cpm) applied to four columns, 1.276 ± 0.026 × 10^6 cpm recovered; and 2.460 ± 0.040 μmole of unlabeled ATP (disodium salt) applied to three columns, 2.456 ± 0.047 μmole recovered. Fraction 1 consisted of the 25 ml eluate of LP; fraction 2 consisted of the 50 ml eluate of UP; and fraction 3 consisted of an additional 50 ml of UP. Values are means ± SEM. Of the crude lipid 50–100 mg was applied to each column.

Discussion. Neutral chloroform–methanol extracts of calcifying cartilage and osseous tissues contain large amounts of nonlipid material, often exceeding in weight the lipid present. Similarly, the nonlipid content of acidic extracts of preextracted calcified tissues was so large that it was difficult to apply enough crude lipid to chromatograms to allow accurate analysis of the constituent phospholipids (Table 2).

Considerable time was spent attempting to achieve success with the Sephadex column method of Wells and Dittmer (4). Apparently the regulation of the flow rate was the critical factor: unless it was very slow, nonlipids were not effectively retarded. Similar difficulty with this method has been experienced by others (6). I suggest that the solvent employed by Wells and Dittmer did not contain sufficient water to form an adequate stationary phase.

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The method reported here provided equally good or better removal of nonlipids, complete recovery of lipid P, and a much faster flow rate, so that purified lipids could be eluted from the columns in 30 min instead of 2–3 hr. An additional advantage is that the columns can be reused after a very simple rinsing procedure.

The present method is in many ways similar to that of Siakotos and Rouser (6), but is simpler to set up and easier to use. In addition, its effectiveness has been validated by different means: recovery of both total and individual phospholipids was checked by phosphorus analysis, and the effectiveness of removal of representative water-soluble nonlipids (inorganic salts, sugars, amino acids, and nucleotides) was evaluated separately. Although the Siakotos and Rouser procedure offers the advantage of being able to separate gangliosides from both water-soluble nonlipids and other lipids, the method offered here provides a useful simplified alternative.

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