Low temperature chromatography of lipids on cellulose

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► The separation of neutral fat from phospholipids has long depended on their solubilities in acetone, and the term "acetone-insoluble lipids" is regarded as synonymous with phospholipids, although the separations obtained are not clear-cut without repeated precipitation. In recent years effective separations have been obtained using chromatography on silicic acid (1, 2), but it is possible that degradation of some of the phospholipids may take place (3, 4). Dialysis through a rubber membrane (5) has been used, but this is slow and is efficient only when required to remove small amounts of neutral fat from the phospholipid fraction (6).

Work in this laboratory (4, 7) has shown that phospholipids after dinitrophenylation and methylation can be divided into an acetone-soluble and an acetone-insoluble fraction. The phosphatidic acidlike phospholipids and those containing a dinitrophenyl group are acetone-soluble, while lecithin and the inositol phospholipids remain acetone-insoluble. The procedure described below has been found to effect a satisfactory separation of these two fractions.

The lipids are dissolved in petroleum ether, b.p. 40°-60°C (approximately 0.5 g. in 100 ml.), the mixture added to 90 g. of cellulose powder ("Whatman's for chromatography"®) and the solvent removed by heating for about 20 minutes at 60°-80°C in a stream of nitrogen to obtain a homogeneous mixture. A slurry of cellulose (30 g.) and acetone is poured into a chromatography column (5 cm. diameter by 35 cm.), the cellulose packed down with the aid of compressed air, and the level of acetone allowed to fall to within 1 cm. of the top of the cellulose. A stopcock at the bottom of the column is turned off and the column placed in a deep-freeze cabinet at −18°C, together with the mixture of lipid and cellulose, and acetone. When equilibrium is attained, the lipid-cellulose mixture is added to the column together with cold acetone, and air bubbles removed by stirring with a glass rod. The stopcock is now opened and 2 l. of cold acetone allowed to flow through the column; this quantity of acetone has been found adequate for complete elution of the acetone-soluble material. The column can then be removed from the deep-freeze cabinet and all the remaining lipids removed by elution with 2 to 3 l. of chloroform-ethanol (2:1, v/v).

It has been found that lower temperatures (−20° to −30°C) do not result in improved separations and that the ratio of acetone-insoluble material to cellulose is critical and should not be more than 1:400. It is also essential that the cellulose should be exhaustively extracted with chloroform before use to remove "lipids" amounting to about 0.1 per cent.

Table 1 gives the results of this separation as applied to dinitrophenylated and methylated phospholipids and it will be seen that the acetone-insoluble material

TABLE 1. Separation of Dinitrophenylated and Methylated Lipids into Acetone-Soluble and Acetone-Insoluble Fractions

Rat Tissue	Total Weight	Acetone-Soluble		Acetone-Insoluble	
		P	DNP*/P	P	DNP*/P
	mg.	*μmole*		*μmole*	
Liver	587	255	0.57	175	0.0
Liver	954†	349	0.57	283	0.0
Heart	487	269	0.53	133	0.038
Kidney	334	125	0.59	75	0.02
Liver mitochondria	306	124	0.55	112	0.012
Liver microsomes	359	120	0.58	150	0.0

* Mole ratio.
† Excluding neutral fat.

contains little or no dinitrophenyl groups while, in the other fraction, the ratio of dinitrophenyl groups to phosphorus (DNP/P) was between 0.53 and 0.59. This fraction contains three main components: phosphatidylethanolamine and phosphatidylserine (DNP/g. atom.P = 1.0), "complex" amino-phospholipids (DNP/g.atom.P. = 0.5), and several components (some unidentified) with no DNP groups (7). Microbiological examination (8) showed that inositol was present only in the acetone-insoluble materials and that mild alkaline hydrolysis yielded glyceryl-phosphorylcholine from the acetone-insoluble materials but none from the acetone-soluble fraction.

The success in separating these phospholipid de-

rivatives prompted an investigation of the separation of neutral fat and phospholipids not dinitrophenylated or methylated. Table 2 shows that the acetone-soluble fraction contained little phosphorus. The percentage of phosphorus in the acetone-insoluble fractions corresponded to the values usually found for phospholipids, except in the case of the brain lipids where the values were consistent with the presence of cerebrosides in the proportions reported in the literature (9). The rat liver lipids, which had been labeled with P^{32} by intravenous injection of $NaH_2P^{32}O_4$ 90 minutes before the animal was killed, yielded acetone-soluble lipids containnig 0.21 per cent P with a specific radioactivity of 80 cpm. per μmole P and yielded acetone-insoluble lipids having an activity of 870 cpm. per μmole P. This demonstrates that the acetone-soluble phospholipids were not merely an adsorbed sample of the total phospholipids. It cannot be decided whether small quantities of acetone-soluble phospholipids were present in each sample or whether in some cases the separations were incomplete.

When lipids are treated with cold acetone, the precipitate contains entrained acetone-soluble compounds, while the supernatant contains acetone-insoluble materials which are solubilized in the high concentrations of neutral fat. Therefore, a clear-cut separation requires a complicated process of fractional precipitation and the smaller the sample, the more difficult it is to obtain a complete separation. It was believed that more efficient separation would be obtained if the lipids were spread out over a large area of cellulose (judged to be a suitable adsorbent) and then washed with cold acetone. The fact that the ratio of acetone-insoluble lipid to cellulose is critical demonstrates that adsorption occurs and that the process is not one of filtration.

In the present investigation the object has been to obtain two fractions only, but some indications have been observed that further fractionation of the acetone-soluble materials is possible. It may be practicable to effect other separations by varying the adsorbent, the solvents, and the temperature. Other fields in which this technique might be useful are the low temperature fractionation of fatty acids and the fractionation of triglycerides in studies of glyceride structure.

TABLE 2. SEPARATION OF NEUTRAL FAT AND PHOSPHOLIPIDS

	Acetone-Soluble			Acetone-Insoluble	
	Weight	P	Total Lipid P	Weight	P
	mg.	*per cent*	*per cent*	*mg.*	*per cent*
Sheep brain	210	0.0084	0.09	592	2.80*
Rat liver	186	0.21	3.3	321	3.55
Hen egg yolk	95	0.015	0.82	48	3.59
Rat brain	109	0.15	1.6	273	2.95
Rat heart	85	0.26	1.9	304	3.73

* Liebermann-Burchard test for sterols was negative.

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