J. Lipid Research, April, 1961
Volume 2, Number 2

Separation of lipid classes by chromatography on florisil*

K. K. Carroll

Collip Medical Research Laboratory,
University of Western Ontario,
London, Ontario, Canada

[Received for publication July 28, 1960]

SUMMARY

Chromatography on Florisil® (activated magnesium silicate) was used to separate model compounds representative of hydrocarbons, cholesterol esters, triglycerides, free sterols, diglycerides, monoglycerides, and free fatty acids. The order of elution was the same as that observed in silicic-acid chromatography except that free fatty acids were eluted after monoglycerides. Recoveries were nearly quantitative and the positions of individual compounds on the chromatograms were highly reproducible. Phospholipids were not eluted under the conditions used for separating the above compounds, and were eluted less readily from Florisil than from silicic acid with methanol. Florisil had definite advantages over silicic acid for the separation of lipid classes by column chromatography. It required no prewashing or other pretreatment except deactivation with water. Columns were quickly and easily packed, and the relatively coarse mesh of the Florisil permitted rapid flow rates. Separations could be achieved in much shorter times with smaller volumes of eluting solvents. Preliminary experiments indicated that chromatography on Florisil gave good separations of lipid classes of naturally occurring lipids extracted from liver and blood.

Current interest in the metabolism of lipids has indicated a need for chromatographic methods of separating the various classes of lipids which occur in nature. Chromatography on silicic acid has been widely used for this purpose since its introduction by Trappe (1, 2) and Borgström (3, 4) but, although it is capable of separating many classes of lipids (5, 6, 7), it has certain disadvantages, and the separations are not always reliable unless particular care is taken to ensure that conditions are duplicated exactly (7).

Chromatography on Florisil® was used in earlier experiments in this laboratory for the separation of fecal sterols from other fecal lipids (8). It had also been previously employed by other workers for the separation and purification of a number of different types of lipids such as tocopherols (9), steroid hormones (10), cerebrosides (11), and fatty acid esters (12). In the course of our experiments it became apparent that the adsorption characteristics of Florisil for classes of neutral lipids were somewhat similar to those of silicic acid. Accordingly, a series of chromatograms was run on Florisil columns with pure reference compounds in order to compare the results with those obtained by other workers using silicic acid. The successful separation of reference compounds led to further experiments on the separation of classes of naturally occurring lipids from rat liver and blood.

* Supported by the Life Insurance Medical Research Fund and by the National Research Council of Canada.

METHODS

Florisil consists of hard, porous, white granules whose composition is reported to be as follows: magnesium oxide 15.5 ± 0.5%, silicon dioxide 84.0 ± 0.5%, and sodium sulfate 0.5% average (1.0% maximum).[1] It was obtained from the Floridin Co. in the form of 60- to 100-mesh material activated at 650°.[2] Material of 100 to 200 mesh was tested in a few experiments, but the coarser grade seemed preferable. Silicic acid (suitable for chromatographic analysis) was obtained from the Mallinckrodt Chemical Works, and Hyflo Super-Cel from the Johns-Manville Co., Ltd.

[1] This information is contained in a bulletin obtained from the Floridin Co., Tallahassee, Fla., on the properties and uses of Florisil as a chromatographic adsorbent.

[2] Florisil activated at 260° or at 110° may also be obtained from the Floridin Co. Chromatographic separations very similar to those described here were obtained by using Florisil activated at 260° without subsequent addition of water. Lipids such as cholesterol palmitate, tripalmitin, and cholesterol were also completely separated on Florisil activated at 110°, but the compounds were eluted somewhat earlier than in the experiments described.

Skellysolve B (a mixture of hydrocarbons, mainly *n*-hexane, boiling at 60°–71°) was obtained from Merck and Co. It was dried over sodium and distilled, and the portion boiling at 68°–70° was used. The purified Skellysolve B will hereafter be referred to as hexane. Merck reagent methanol and chloroform and Mallinckrodt anhydrous ether were used without further purification.

Standard reference compounds were obtained as follows: Paraffin (refined, m.p. 53°–55°)—A. H. Thomas Co.; squalene—Matheson, Coleman and Bell; cholesteryl palmitate—a gift of Dr. Leon Swell, Veterans Administrative Center, Martinsburg, West Virginia; tripalmitin—Hormel Institute, Austin, Minnesota; cholesterol—a commercial preparation purified by two recrystallizations from ethanol; 1,2-dipalmitin and 1-monopalmitin—gifts of Dr. F. H. Mattson, Procter and Gamble Co., Cincinnati, Ohio; palmitic acid—Nutritional Biochemicals. Rat liver and blood lipids were obtained by repeated extraction with ethanol-ether 3/1 (v/v) at room temperature. The extracts were concentrated *in vacuo* and the crude lipids were re-extracted from the concentrate with petroleum ether and dried over sodium sulfate.

Column Preparation. The initial experiments were carried out with a column containing 30 g of Florisil and measuring 2.0 cm in diameter × 17 cm in length. It was prepared by filling the chromatographic tube with hexane and adding the dry Florisil in portions, tapping the tube between additions to ensure even packing. Later experiments were carried out with a 12 g column, measuring 1.2 cm × 15 cm, prepared in the same way.

The early experiments were performed with a sample of Florisil which had been in the laboratory for several years. This gave satisfactory separations when used without preliminary treatment, but when similar separations were attempted with a fresh supply of Florisil, it was found that the different lipid classes were eluted much later in the chromatogram. This indicated that the new Florisil had a greater adsorption strength. It was therefore deactivated by mixing with a measured volume of water in a glass-stoppered round-bottom flask, shaking until the mixture was free-flowing, and allowing to stand overnight to equilibrate. It was then packed in a column as described above. The addition of 7 ml of water per 100 g of Florisil gave material which had chromatographic properties similar to the Florisil used in the initial experiments.

Chromatographic Procedure. When all the Florisil had been added to the column, the excess hexane was allowed to flow out until the surface of the liquid reached the top of the packed column, and the lipids to be separated were added to the top of the column in a small volume of hexane. The head of liquid was again allowed to drop to the top of the column, and elution was carried out as described for individual experiments. Reference compounds such as mono- and diglycerides, which were relatively insoluble in hexane, were added as dry powders to the top of the column.

During elution of the columns bubbles of solvent vapor occasionally formed, and in some cases caused actual breaks in the column. This tendency increased as the proportion of ether in the eluting solvent increased, but it was generally not serious provided the surrounding temperature was below 28°. The column temperature could be controlled by using jacketed columns, but this was not done in the present experiments and in our experience the chromatographic separations were not appreciably affected by variations in room temperature.

Analysis. The eluent from the columns was collected in 5 or 10 ml fractions, and the distribution of lipids was determined gravimetrically after the lipid residues from individual fractions had been transferred with a minimum of solvent to tared glass tubes. The position of individual reference compounds was determined by running a separate column for each compound. The distribution and recovery were also checked in some cases by the use of colorimetric methods. Cholesterol was measured by the method of Sperry and Webb (13). Glycerol was determined on hydrolysis products of the lipids by the method of Lambert and Neish (14) as modified by Korn (15). Phosphorus was measured by the method of King (16) after oxidation of the lipids with perchloric acid.

RESULTS

Large Florisil Column (30 g). The separation of a cholesterol ester, a triglyceride, free cholesterol, a diglyceride, and a monoglyceride is illustrated in Figure 1. The sequence of eluting solvents used in this chromatogram (Table 1) gave complete separation and nearly quantitative recovery of the five different compounds. The use of 10% methanol in ether as the final eluting solvent gave a sharp peak of monoglyceride, but also eluted extraneous material from the column as shown by a smaller peak following the monoglyceride. In subsequent work this was avoided by eluting the monoglyceride with 2% methanol in ether. Other experiments showed that much larger amounts (at least threefold) of the reference compounds could be separated without overlap on this column. It therefore seemed desirable to devise an elution procedure for a smaller column in order to achieve the same separation

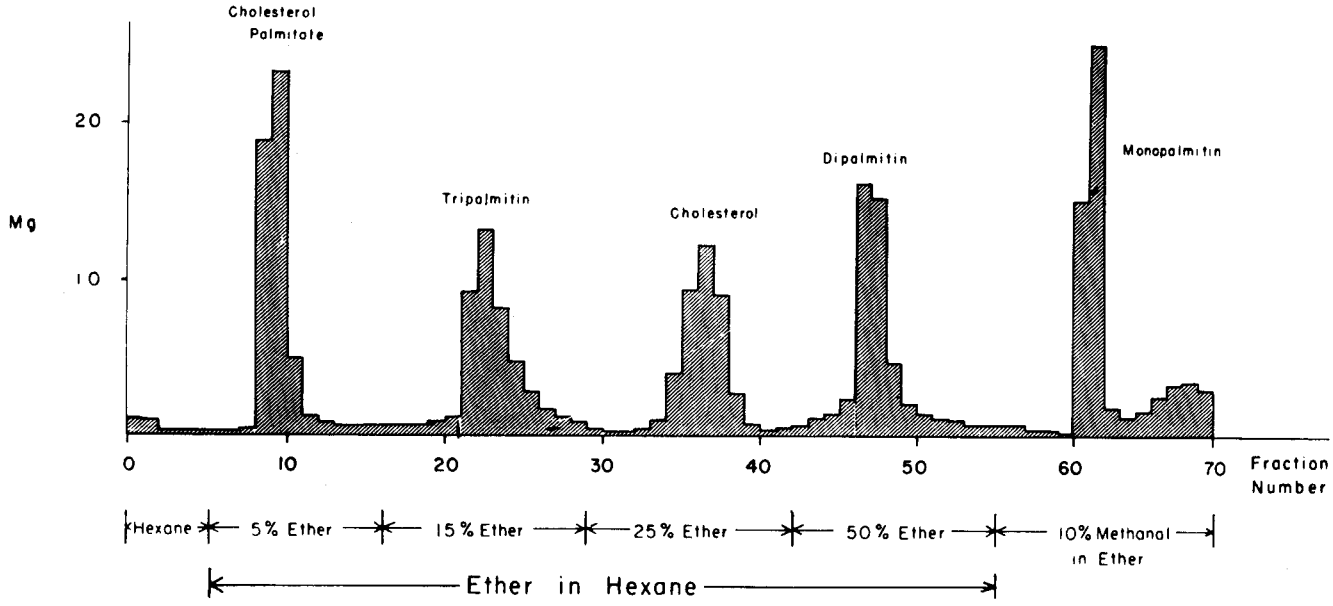

FIG. 1. Separation of lipid classes on 30 g column of Florisil. Ten ml fractions were collected. The column load consisted of 40 mg each of cholesterol palmitate, tripalmitin, cholesterol, 1,2-dipalmitin, and 1-monopalmitin.

with considerable saving in solvents and in the time involved.

Small Florisil Column (12 g). Chromatograms on this column were nearly all carried out with a new supply of Florisil which required deactivation with water for satisfactory separation of the reference compounds. Figures 2 and 3 illustrate the differences obtained when the Florisil was used with no added water, and with 7% added water. In the experiment shown in Figure 2, each of the compounds emerged much later from the chromatogram, the triglyceride overlapped the cholesterol, the diglyceride was only partially eluted, and the monoglyceride remained on the column. However, when the Florisil was hydrated with 7% water (Fig. 3), the chromatographic pattern was similar to that obtained with the large column (Fig. 1). In Figure 3 cholesterol and dipalmitin were both eluted with 25% ether in hexane. This gave a broader diglyceride peak than if the dipalmitin were eluted with 50% ether, but for practical purposes it was an advantage to eliminate the solvent change.

TABLE 1. ELUTION SCHEDULES FOR FLORISIL CHROMATOGRAMS

Eluent	30 g Column (2.0 cm × 17.0 cm)	Eluting Solvent	12 g Column (1.2 cm × 15.0 cm)
	ml		*ml*
Hydrocarbons	50	Hexane*	20
Cholesterol esters	120	5% ether in hexane	50
Triglycerides	150	15% ether in hexane	75
Cholesterol	150	25% ether in hexane	60†
Diglycerides	150	50% ether in hexane	60†
Monoglycerides	150	2% methanol in ether	75
Free fatty acids	150	4% acetic acid in ether	75

* Purified Skellysolve B.

† It may be found more convenient to elute both cholesterol and diglyceride fractions with 140 ml of 25% ether in hexane.

It may be noted that the degree of hydration of the Florisil had a greater effect on the position of triglycerides and diglycerides in the chromatogram than it did on the position of cholesterol. When the Florisil was hydrated with less than 6% water, the tripalmitin tended to trail into the cholesterol fraction. When it was hydrated with more than 6% water there was a tendency for the cholesterol and dipalmitin fractions to overlap. Hence, 7% hydration appeared to be an acceptable compromise, although the exact amount of water used was not critical.

Differences in degree of hydration of the Florisil also affected the separation of paraffin hydrocarbons and squalene. Both were eluted before cholesterol esters

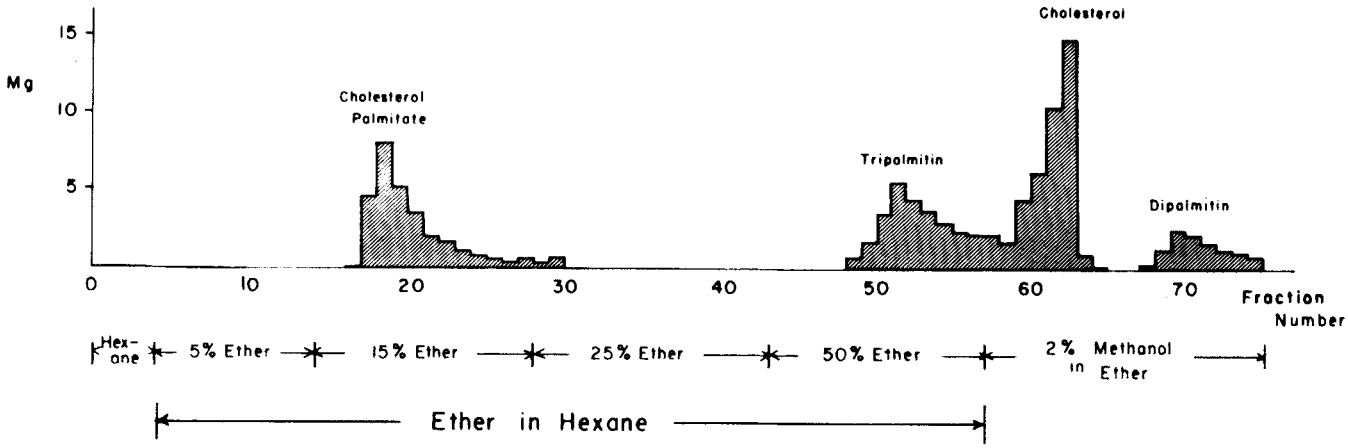

FIG. 2. Separation of lipid classes on 12 g column of highly active Florisil. Five ml fractions were collected. The column load consisted of 30 mg each of cholesterol palmitate, tripalmitin, and cholesterol, and 15 mg each of dipalmitin and monopalmitin. The amounts of eluting solvents were in some cases greater than those listed in Table 2.

on a column of Florisil hydrated with 7% water, but they were incompletely separated from one another (Fig. 4). However, on a column of Florisil with no added water the hydrocarbon and squalene fractions were completely separated (Fig. 5).

Free fatty acids were retained on the Florisil column during the elution with neutral solvents up to and including 2% methanol in ether, although in silicic-acid chromatography free fatty acids are eluted immediately after the triglyceride fraction (7). It seems likely that this difference is due to the basicity of Florisil, since palmitic acid was quantitatively eluted as a sharp peak from a Florisil column hydrated with 7% water by using 4% acetic acid in ether as the eluting solvent. The eluted material was very soluble in ether, and its melting point indicated that it was free palmitic acid rather than the magnesium salt. Small amounts of ether-insoluble material were also eluted with 4% acetic acid in ether, but whether this was magnesium acetate or some other substance was not determined.

In the results presented in Figures 1 to 5 the flow rate was controlled with a stopcock to 80 to 100 ml/hour, but in one experiment a mixture of cholesterol palmitate, tripalmitin, and cholesterol was eluted from a 12 g column at the maximum flow rate (160 to 180 ml/hour), and the separation of components was the same as that obtained at slower flow rates. Even at the controlled flow rate Florisil offers definite advantages over silicic acid in terms of saving time and material, as is indicated by the following figures. The preparation and elution of a 12 g column of silicic acid took, in our experience, approximately 24 hours and required about

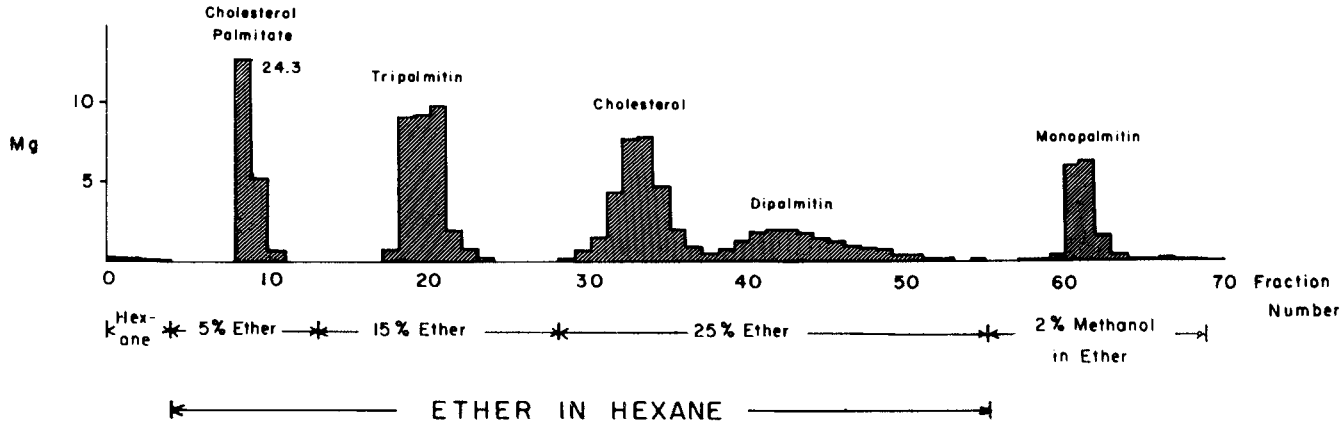

FIG. 3. Separation of lipid classes on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load was the same as in Fig. 2.

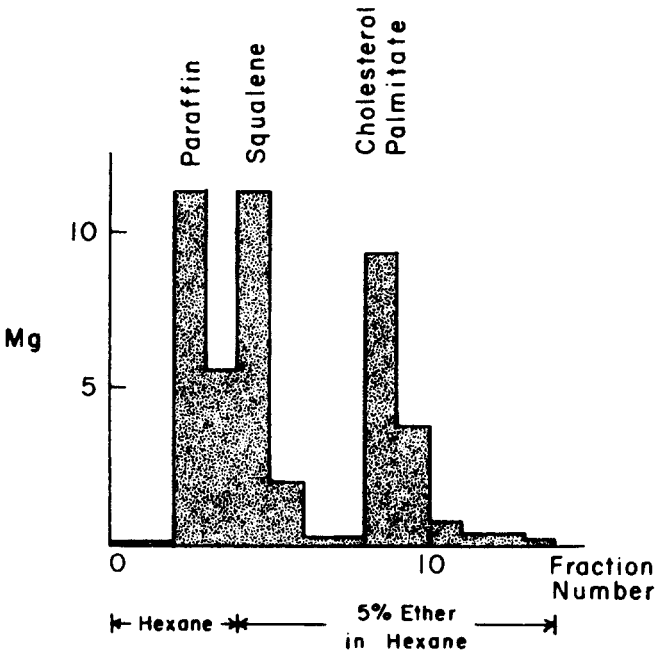

FIG. 4. Separation of a mixture of 15 mg each of paraffin, squalene, and cholesterol palmitate on a 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected.

1000 ml of solvent. A similar separation of neutral lipids was achieved on a 12 g column of Florisil in less than 4 hours with 350 ml of solvent.

The recovery of model compounds from Florisil columns was always greater than 90%, and in most cases was between 95% and 105%. Some of this variation was undoubtedly due to experimental errors in weighing.

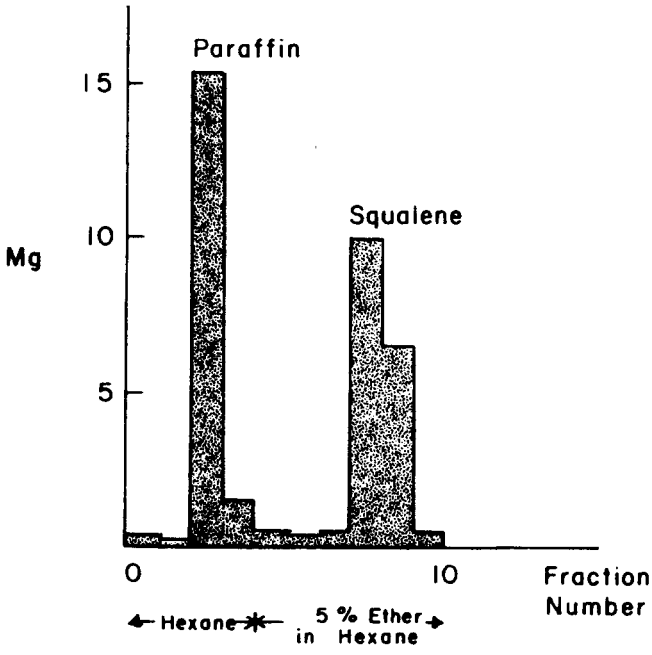

FIG. 5. Separation of a mixture of 15 mg each of paraffin and squalene on highly active Florisil. Five ml fractions were collected.

Separation of Naturally Occurring Lipid Mixtures. A number of chromatograms were run on 12 g Florisil columns with lipid extracts of normal rat liver (Fig. 6). Definite peaks corresponding to cholesterol ester, triglyceride, and free cholesterol fractions can be seen, and an analysis of these fractions for cholesterol and for glycerol indicated that the peaks actually contained the expected class of compound and that there was little or no overlapping of one class of compound with another. A similar chromatogram on rat blood lipids also gave major peaks corresponding to cholesterol esters, triglycerides, and free cholesterol (Fig. 7).

Phospholipids were not eluted with the solvents listed in Table 1. However, an attempt was made to separate the neutral lipids and phospholipids of rat liver by chromatography on a Florisil column under conditions similar to those used by Borgström with silicic acid (3). Five g of Florisil were added in portions to a column 1 cm in diameter filled with chloroform. A similar column containing 5 g of silicic acid and 2.5 g of Hyflo Super-Cel was prepared in chloroform as described by Hanahan *et al.* (17) and used as a control. Equal aliquots of a liver lipid extract dissolved in chloroform were applied to each of these columns. They were eluted first with 100 ml of chloroform and then with three 50 ml portions of methanol. As shown in Table 2, the phospholipid was recovered quantitatively in the first 50 ml of methanol from the silicic-acid column, while less than half of the total phospholipid was recovered from the Florisil column with 150 ml of methanol. The results shown in Table 2 suggested some fraction of phospholipids on the Florisil column, and further experiments were carried out in which smaller fractions were collected, but these indicated

TABLE 2. ELUTION OF LIVER PHOSPHOLIPIDS FROM SILICIC ACID AND FROM FLORISIL*

	5 g Silicic Acid 2.5 g Hyflo Super-Cel	5 g Florisil
Eluent *ml*	Phosphorus *μg*	Phosphorus *μg*
100 chloroform	8	4
50 methanol	1800	540
50 methanol	80	96
50 methanol	35	224

* Column load: 5 ml of chloroform containing approximately 55 mg of total lipid and 1800 μg of phosphorus.

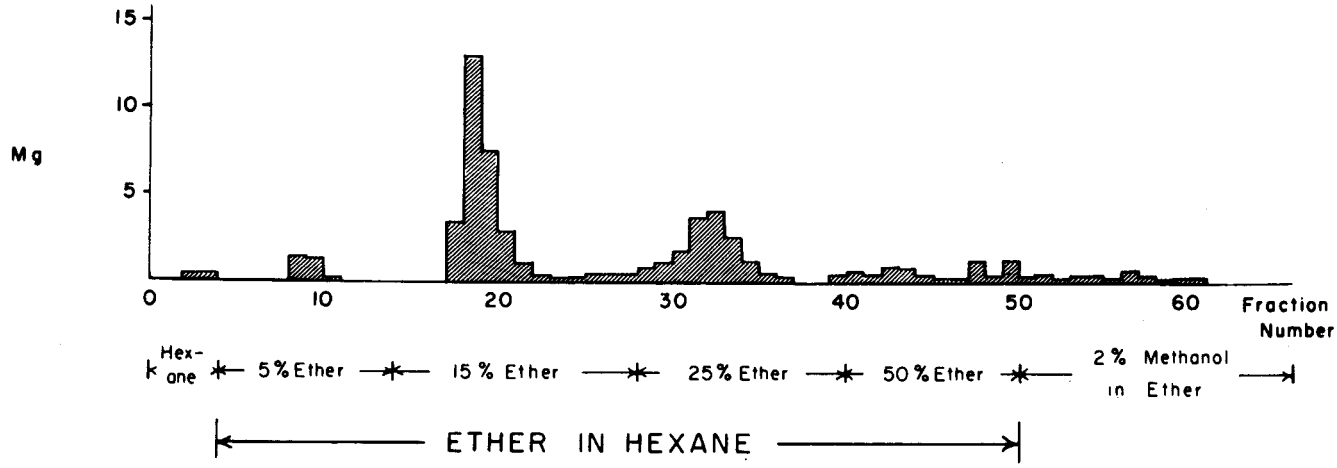

FIG. 6. Separation of rat liver lipids on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load consisted of 245 mg of crude lipid extract.

that Florisil was probably not useful for the fractionation of phospholipids. Phosphorus was present in each of the small fractions and no sharp peaks were evident.

DISCUSSION

The results of this study showed that chromatography on Florisil could be used to separate classes of neutral lipids in much the same way as chromatography on silicic acid, and that use of Florisil rather than silicic acid offered a number of distinct advantages. Florisil columns were very simple to pack, the material required no prewashing, and the relatively coarse mesh used for these experiments permitted rapid flow rates. Florisil chromatography had the further advantage that free fatty acids were eluted after the various glyceride fractions, and this eliminated the possibility of their overlapping with triglycerides or diglycerides (5, 7).

The degree of hydration of the Florisil was shown to affect its chromatographic properties markedly, and, furthermore, its adsorption affinity for some types of lipids was affected more than that for others (Figs. 2 to 5). Hence, desired separations could be facilitated by using Florisil with the appropriate degree of hydration.

It seems probable that the highly active Florisil absorbs moisture from the atmosphere unless it is kept under anhydrous conditions. This may explain why the Florisil which was stored in the laboratory for several years differed in its adsorption properties from that obtained later. However, the original lot of Florisil did not alter noticeably during the period of months over which it was used, although the weather was very humid for part of the time and no special attempt was made to protect the Florisil from the atmosphere. This suggested that it may have reached an equilibrium condition in which it had little tendency to absorb more water. It therefore seems desirable either to store the

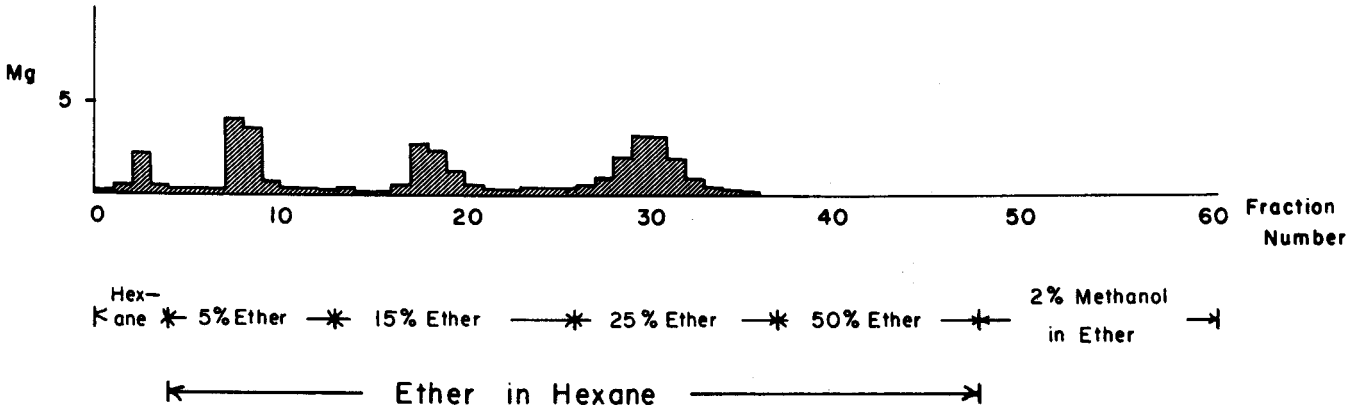

FIG. 7. Separation of rat blood lipids on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load consisted of 80 mg of crude lipid extract.

Florisil in the hydrated state or to maintain the highly active Florisil under anhydrous conditions in order to ensure consistent chromatographic results.

In this work the eluent from the chromatograms was collected in small fractions in order to determine the sharpness of the peaks and the degree of separation of individual compounds or groups of compounds. In our experience, the position of any given compound on the chromatogram was highly reproducible. Therefore, in studying naturally occurring lipid mixtures, it would probably be satisfactory to collect larger fractions in such a way that each fraction contained a major class of neutral lipid. It should not, however, be assumed that the position of a compound on the chromatogram is a guarantee that it belongs to a certain class of lipid. For example, it was found in other unpublished experiments that fatty alcohols were eluted together with triglycerides. It has been shown that the nature of the fatty acid moiety may affect the position on silicic-acid chromatograms of lipids belonging to a given class (7), and this applies also to Florisil chromatograms (12). Therefore, positive identification of lipids eluted from Florisil can be made only on the basis of further characterization.

The author is greatly indebted to Mrs. Fannie Angeles, Miss Kay Parkes, and Mr. E. Pedersen for their capable technical assistance during the course of this work.

REFERENCES

1. Trappe, W. *Biochem. Z.* **306:** 316, 1940.
2. Trappe, W. *Biochem. Z.* **307:** 97, 1941.
3. Borgström, B. *Acta Physiol. Scand.* **25:** 101, 1952.
4. Borgström, B. *Acta Physiol. Scand.* **25:** 111, 1952.
5. Fillerup, D. L., and J. F. Mead. *Proc. Soc. Exptl. Biol. Med.* **83:** 574, 1953.
6. Barron, E. J., and D. J. Hanahan. *J. Biol. Chem.* **231:** 493, 1958.
7. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1959.
8. Carroll, K. K. *J. Lipid Research* **1:** 171, 1960.
9. Devlin, H. B., and H. A. Mattill. *J. Biol. Chem.* **146:** 123, 1942.
10. Nelson, D. H., and L. T. Samuels. *J. Clin. Endocrinol. and Metabolism* **12:** 519, 1952.
11. Radin, N. S., F. B. Martin and J. R. Brown. *J. Biol. Chem.* **224:** 499, 1957.
12. Kishimoto, Y., and N. S. Radin. *J. Lipid Research* **1:** 72, 1959.
13. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
14. Lambert, M., and A. C. Neish. *Can. J. Research* **28B:** 83, 1950.
15. Korn, E. D. *J. Biol. Chem.* **215:** 1, 1955.
16. King, E. J. *Biochem. J.* **26:** 292, 1932.
17. Hanahan, D. J., J. C. Dittmer and E. Warashina. *J. Biol. Chem.* **228:** 685, 1957.