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Isolation and determination methods for brain cerebrosides, hydroxy fatty acids, and unsaturated and saturated fatty acids *

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SUMMARY

A new method is described for the isolation of nearly pure total cerebrosides from fresh brain. Florisil columns are used to separate the cerebroside hydroxy and normal acids, in the form of their esters, and to separate the saturated and unsaturated esters of each group. The esters within each class are determined by gas chromatography, the hydroxy esters being run as the methyl ethers. Yields in the various steps are close to theoretical. The presence of lower homologues in all four classes of rat brain cerebroside acids was discovered. Particularly large amounts of C_{23} and C_{22} saturated acids were found.

Although the major fatty acids occurring in brain cerebrosides were identified over 30 years ago, mainly through the efforts of Klenk and Thierfelder, as reported by Deuel (1), the percentage of each acid has never been determined except in a rough way by fractional isolation of the different cerebrosides (2). To make such an analysis it is necessary to isolate the cerebrosides quantitatively, so that no enrichment of one type occurs during the isolation, and in pure form, free from other compounds containing fatty acids. The analysis of the fatty acids from cerebrosides is a problem in itself, complicated by the presence of very lengthy chains and hydroxy groups.

This paper describes the isolation of cerebrosides by an extension of our previously described method (3), in which the lipids of whole brain are applied to a Florisil [1] column, the cholesterol is eluted with ether, and the cerebrosides are eluted with chloroform-methanol. A contaminating ester (4) is saponified with alkali, and the reaction products are removed with water and ion exchange resins. The cerebroside sulfate is removed in the latter step.

The cerebroside acids are liberated by methanolysis and the esters are separated by chromatography into two groups: normal and hydroxy esters. The two groups are each divided into saturated and unsaturated esters by formation of mercurial addition products and Florisil chromatography. The four groups of esters are then analyzed by means of gas chromatography.

It is believed that the methods described here are of use in other analytical and isolation problems involving cerebrosides and fatty acids.

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[1] Floridin Co., Tallahassee, Fla.

EXPERIMENTAL

Materials. A simple glass still was used to redistill nearly all the solvents: ACS absolute methanol, USP chloroform, ACS toluene, 95 per cent ethanol, and petroleum ether b.p. 66-7°C (Skellysolve B). Absolute ether, used for chromatography, was freshly redistilled from KOH pellets; USP ether was used for extractions.

Florisil, purchased as 60 to 100 mesh, was resieved to 70 to 100 mesh and stored in glass bottles. If the sieving is done in hot humid weather, adsorptive power is partially lost and reactivation is necessary. This is done by heating the adsorbent 1 hour at 650°C. Dowex 1-X2 anion exchange resin (200 to 400 mesh) was cycled and converted to the hydroxyl form; Dowex 50-X4 (200 to 400 mesh) was cycled

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and converted to the hydrogen form. The resins were mixed in the proportion 7:2, based on moist weight.

Lignoceric acid was prepared by the method described by Carroll (5), and from this, DL-cerebronic acid (m.p. 97°-98°C), by bromination and hydrolysis (6). The previously reported melting point for DL-cerebronic acid was 99.5°-100°C (7). Our preparation of methyl DL-cerebronate melted at 74.5°-76°C, in contrast to the reported melting point of 65°C for the D-cerebronate (8). Samples of lignoceric acid, nervonic acid, and methyl α-hydroxybehenate were generously supplied by Dr. K. K. Carroll. Pure methyl oleate, palmitate, and stearate were obtained from the Hormel Foundation.[2] Commercial grades of α-hydroxystearic, arachidic, and behenic acids and also methyl myristate and laurate were used as identification standards for gas chromatography.

Equipment. The column techniques were described previously (9). Evaporations were performed under vacuum with a swirler evaporator (10) or rotary evaporator. Gas chromatography was performed with an Aerograph fitted with a thermal conductivity detector and a 30-inch copper column (¼ inch O.D.).[3] The packing for the column contained the polytetramethylene glycol-succinate ester described by Dr. B. M. Craig, to whom we are indebted for information about this material. The samples of esters for analysis were dissolved in about 3 volumes of xylene and about 10 microliters of solution were injected into the column with a micro-syringe.[4]

Isolation of Crude Cerebrosides. A group of from 11 to 30 rat brains was homogenized with 20 volumes of chloroform-methanol (2:1) according to the method of Folch *et al.* (11); the mixture was filtered, and the extract was taken to dryness in a rotary evaporator. Toluene was added near the end of the evaporation to prevent splashing. The lipids were dried overnight in an evacuated desiccator over KOH and wax. To ensure cleavage of the proteolipids, the extract was refluxed 45 minutes with six volumes of chloroform-methanol and filtered, evaporated to dryness, and again dried in a desiccator.

From 45 to 120 grams of Florisil (30 g. per g. of lipid) were swirled briefly under vacuum with absolute ether, and the slurry was poured into a column 2.2 to 2.8 cm. I.D., yielding a packing about 45 cm. high. The crude lipids were warmed with ether (40 ml. per g. of lipids), and the suspension was swirled under vacuum with more Florisil (10 g. per g. of lipids). This mixture was added to the column, together with two rinses of ether, and elution was continued with the same solvent (1,000 ml. per g. of lipids). This fraction includes the lipids of low polarity, particularly cholesterol.

The crude cerebrosides were next eluted with chloroform-methanol 4:1 (1,000 ml. per g. of lipids). Evaporation to dryness yielded a white powder which took on a slight yellow tint on storage in a desiccator.

The flow rate was 400 to 500 ml. per hour for an 80 g. column (2.2 cm.). In early experiments we found that the column tended to form bubbles, but this was prevented by applying slight air pressure on top and a slight constriction below (with Neoprene tubing and a screw clamp). Addition of the lipids suspended in ether, without Florisil, resulted in clogging and bubble formation. Stopping the column flow for any extended period also tends to produce bubbles.

Removal of Ester and Ionic Lipid. The crude cerebrosides were dissolved in 450 volumes of methanol (based on cerebroside weight), and 45 volumes of aqueous NaOH (40 g. per 45 ml. water) were added with cooling. After stirring and heating at 37°-39°C for 3 hours, phenol red was added, and then concentrated HCl (with cooling) until just past the orange end point. The mixture was evaporated to dryness and dried over P_2O_5. The residue was extracted with warm chloroform-methanol (2:1) while crushing the salt cake with a glass rod, the salt was filtered off, and the filtrate was washed with water in a centrifuge tube to extract the alcoholic portion of the ester. Probably some cerebroside sulfate was lost in the water (12).

The chloroform and interface material were evaporated to dryness, the lipids were dissolved in ethanol-toluene-water (7.5:6.1), and the ionic lipids were removed by passing the solution through a column of mixed ion exchange resins. The column packing was 1 × 35 cm.; the resins were prewashed in the column with 250 ml. of the same solvent; the sample was added in the course of 1 hour, and elution was carried out with 250 ml. of the same solvent in the course of 1 to 2 hours. The effluent was evaporated to dryness and dried further in a desiccator. Samples of these cerebrosides were drawn for galactose determination by the phosphoric acid-anthrone method (3).

Methanolysis and Separation of Normal and Hydroxy Acids. The cerebrosides (130 to 300 mg.) were dissolved in 20 ml. of Mg-dried methanol saturated with HCl at 0°C, and refluxed 16 hours with an oil bath set at about 95°C. The resultant esters were extracted with four 15 ml. portions of Skellysolve B, 30 ml. of ether were added to the pooled extracts, and the solution was washed with water five times. The yield of

[2] Austin, Minn.
[3] Wilkens Instrument and Research, Inc., Walnut Creek, Calif.
[4] Hamilton Company, Whittier, Calif.

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dry esters ran a few per cent too high, probably because of the extraction of some sphingosine derivatives.

Eight grams of Florisil in Skellysolve B were packed into a 1.1 cm. I.D. column, and the mixed esters were added in 20 ml. of the same solvent. Elution with 1,600 ml. of the petroleum ether yielded the normal (unhydroxylated) esters. The hydroxy esters were eluted with 1,200 ml. of Skellysolve B-absolute ether (9:1). The flow rate was about 400 ml. per hour. The two effluents were evaporated to dryness, dried further under vacuum, and weighed.

Separation of Saturated and Unsaturated Esters. The dry esters were heated in a screw-cap test tube (Teflon-lined cap) for 2 hours at 66°C with about four mole equivalents of mercuric acetate and 15 volumes of methanol (based on the weight of the acetate). The mercuric acetate was first dried over P_2O_5. Under these conditions the unsaturated esters form the following polar grouping: —CH(HgOAc)—CH-(OCH_3)— by addition to the double bond (13). The reaction mixture was transferred to a separatory funnel with 50 ml. of ether and washed with two portions of acetic acid-water (1:30). Five additional washes with water completed the removal of the excess reagents. The ether layer was evaporated to dryness and stored in the dark over P_2O_5. The other steps were carried out under reduced light conditions.

In the case of the normal esters, the sample was dissolved in 5 ml. of Skellysolve and passed through a 0.6 cm. I.D. column containing 2 g. of Florisil in the same solvent. Elution with 410 ml. of Skellysolve yielded the saturated esters, and the unsaturated esters were eluted with 50 ml. of ethanol-chloroform-concentrated HCl (10:8:1). The HCl re-forms the double bond in the original configuration (14), and the polar solvent readily elutes the ester. To minimize the possibility of hydrolysis of the ester by exposure to acid, we caught the effluent in a flask containing 70 ml. of water and 50 ml. of ether. The organic layer was washed well with water to remove mercuric salts and also a good deal of inorganic material arising from the Florisil ($MgCl_2$?). The esters were dried and weighed. In the case of the hydroxy esters, the saturated esters were eluted with Skellysolve-ether (9:1).

Gas Chromatography of the Esters. The above separations into four groups of esters were carried out because the column that was available could not separate the individual esters sufficiently well. It was found that the hydroxy esters did not come off the column at the maximum temperature attempted (217°C), so the methyl ethers were prepared and found to give satisfactory curves. The C_{22} methoxy ester came off the column at the same time as the C_{24} normal ester.

Methylation was performed by refluxing about 35 mg. of hydroxy ester with 1 ml. of redistilled methyl iodide and 200 mg. of dry silver oxide. The mixture was stirred with a magnetic stirrer and heated with a water bath. After 30 minutes another portion of iodide and oxide was added, and then, after 60 minutes more, another portion of oxide. Heating and stirring were continued for a total period of 3 hours, toluene was added, the mixture was filtered, and the solids were washed well with toluene. The filtrate was evaporated to dryness and dissolved in xylene for injection into the column. The ethers were chromatographed at about 216°C and the normal esters at about 206°C. The helium flow rate was about 105 ml. per minute for the ethers and 90 ml. per minute for the normal esters.

RESULTS AND DISCUSSION

Isolation of Purified Cerebrosides. When the cerebroside elution from Florisil is followed by examination of successive portions of effluent, it is seen that the content of white solid (crude cerebrosides) decreases by long trailing to a very low level. At this point a small amount of oily material starts to come off, with perceptible overlapping. In the procedure used for this study, some of this oil is included in the crude cerebrosides. The elution of the oil is greatly speeded by use of chloroform-methanol (2:1). The yield of oil was 12.8 mg. per brain in 81 g. rats, and 17.5 mg. per brain in 449 g. rats, so it can be seen that this fraction is one of the more plentiful brain lipids. The oil and the ether effluent from the oldest group of rats were hydrolyzed with 2 N HCl and the galactose contents determined by an anthrone procedure (15). No galactose was found in the ether effluent, but an amount corresponding to 3.4 per cent of the cerebroside galactose was found in the oil. It is likely that this glycolipid is not trailing cerebroside, but rather Fraction III isolated by Weiss (16). Fraction III, for which we suggest the name neuroside, is a glycolipid which strongly resembles a cerebroside but is more polar, and contains an amino compound which becomes water-soluble on acid hydrolysis.[5]

The use of saponification for further purification of the crude cerebrosides was prompted in part by our observation that a degradation product formed during purification of cerebrosides by reaction with $Ba(OH)_2$ (15), and in part by the observation of an ester ab-

[5] Unpublished work, N. S. Radin and M. V. Kelley, Jr.

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sorption peak by Burton *et al.* (4). The saponification step was investigated with conditions of increasing severity, using a sample of crude cerebrosides from spinal cord (15) and evaluating the results from the yields of cerebrosides and their galactose contents. The very mild conditions of Dawson (17) were inadequate, and we turned to the use of almost 2 N NaOH. Heating 1, 3, and 6 hours resulted in yields of 77.6, 76.7, and 75.0 per cent, while the galactose content of all three samples was very similar. Apparently there is a very slow breakdown of cerebrosides (psychosine would stick to the ion exchange resins), so a 3-hour saponification was accepted as a reasonable time. The cerebroside sulfate that is present in the crude cerebrosides does not lose the sulfate group during saponification; such a reaction would show up as an increasing rather than decreasing yield of cerebroside. We had previously shown the efficient uptake of lipid sulfates by ion exchange resins with the aid of sulfur-labeled lipids (12). The galactose contents of the crude and purified cerebrosides were compared in the cases of two groups of rats, and the differences in contents were calculated as cerebroside sulfate (Mg salt). The sulfatide contents per brain came out 2.25 and 9.0 mg. for groups B and D (see accompanying paper). The sulfatide to cerebroside ratios are 0.25 and 0.41, in reasonable agreement with the estimate made by Blix (18).

The effectiveness of the ion exchange resins for removing the fatty acids resulting from saponification was tested by addition of C^{14}-palmitic acid to the saponification mixture. Using a column of resins 0.6 $\times$ 15 cm., it was found that 4.2 per cent of the radioactivity leaked through, so the amount of resins was raised almost sevenfold. Later work with this palmitic acid showed that the leaking material was a nonionic radioactive impurity.

The galactose contents of the resultant cerebrosides in a series of five isolations ranged from 20.6 to 21.4 per cent. The theoretical galactose content, assuming (19, 20, 21) the cerebrosides are monohydrates of phrenosine (21.3 per cent galactose) and kerasine (21.7 per cent galactose) in the ratio 60:40, is 21.46 per cent. Allowing for the presence of shorter fatty acids might bring this up to 21.6 per cent. On this basis our preparations were 95 to 99 per cent pure. The nature of the impurity is unknown.

The actual yield of cerebrosides was not rigorously determined, but it appears to be practically quantitative. Previous work with brain cerebrosides (3) had shown that there is complete recovery when the Florisil is eluted with chloroform-methanol (2:1). Complete recovery is also obtained when a sample of cerebrosides is reprocessed through the saponification and ion exchange steps.

The Methanolysis Step. As indicated by Rosenheim (20), methanol-sulfuric acid was found to yield free acid as well as methyl esters. Allowing the refluxing vapors to percolate through silica gel to remove water prevented formation of free acids but the yield of esters was low. Possibly the esterification of methanol with sulfuric acid is a strongly competing reaction. After studying various conditions, it was decided that a lengthy treatment with maximal HCl concentration in methanol was best. This gives too high a yield of petroleum ether-extractable lipid, but the excess material is strongly adsorbed by Florisil and is separated from the esters when the normal and hydroxy esters are separated. Thus the yields of crude esters from five rat groups were 54.0, 52.5, 52.9, 50.2, and 52.6 per cent of the cerebroside weights. After chromatography, the normal plus hydroxy esters amounted to 45.7, 45.7, 46.3, 48.9, and 47.6 per cent of the cerebroside weights. The expected value is about 47 per cent.

Separation of Normal and Hydroxy Esters. Early attempts at separating the normal and hydroxy esters by converting the latter to hemisuccinates (22) or sulfates (23) and separating the derivatives by solvent partition led to incomplete separation. Florisil was found to be a suitable adsorbent and was tested with separate esters (stearate, lignocerate, cerebronate, and hydroxystearate) and with known mixtures. The maximal loading of hydroxy esters is roughly 10 to 15 mg. per g. of adsorbent; with higher loads some leakage into the normal ester fraction is observed. The recoveries are excellent provided the long trailing typical of Florisil is overcome by adequate volumes of eluting solvent. The trailing should be reduced by use of finer mesh powder. A small amount of material comes off the adsorbent, but this is largely left behind on the glassware when the effluent is evaporated to dryness and the esters are redissolved in petroleum ether and transferred. The blank thus obtained usually is about 0.05 mg. per g. of adsorbent.

Of general interest is the finding that cholesterol is eluted with the hydroxy esters. This means that Florisil can be used to replace alkaline saponification for the separation of cholesterol from fatty acids, provided there is no appreciable amount of hydroxy fatty acid present. In other words, one can split a lipid sample by methanolysis, extract the esters and cholesterol with petroleum ether, and separate the fatty esters and cholesterol with a Florisil column. Where very long fatty acids are present (such as lignoceric), or excessive emulsification and pigment formation occur, this method is superior to solvent separation of "sa-

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TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF KNOWN ESTER MIXTURES

	Palmitate	Arachidate	Lignocerate
	percentage	*percentage*	*percentage*
Mixture 1			
Known composition	23.0	22.8	54.2
Observed compositions	24.4	23.8	52.0
	23.6	24.8	51.6
	24.6	22.9	52.5
Mixture 2			
Known composition	18.6	40.8	40.6
Observed compositions	18.2	41.0	40.8
	18.6	40.5	40.9
Mixture 3			
Known composition	23.5	55.4	21.1
Observed compositions	23.4	54.5	22.2
	22.9	56.0	21.1

ponifiables" and "unsaponifiables." Free fatty acids and most pigments are adsorbed very strongly by Florisil and can be separated from cholesterol.

Separation of Saturated and Unsaturated Esters. The use of the mercury addition products for separation of these esters by paper chromatography was described by Inoue *et al.* (24), and very recently a note has appeared describing the use of a silica gel column (14). Our method was checked [6] with 48.3 mg. of pure methyl oleate and a 3-g. Florisil column. The yield of material coming off with petroleum ether was 0.9 mg. (corrected for a blank of 0.56 mg.), and the yield of material eluted by the acidic solvent was 47.7 mg. (99 per cent). Assay by a modified hydroxamic acid method (25) indicated the product was 97.4 per cent ester.

Another test, with 25.2 mg. of methyl cerebronate resulted in recovery in the hydroxy ester fraction of 24.8 mg. of material having the original melting point. Apparently our procedure results in losses of about 2 per cent.

Preparation of the Methyl Ethers. A test run with 111.2 mg. of methyl DL-cerebronate resulted in a yield of 115.3 mg. of ether (100 per cent). Of course a weight measurement gives an unprecise indication of completeness of methylation. A methoxyl determination on this material [7] gave a value of 14.61 per cent methoxyl (theoretical is 15.01 per cent). Two recrystallizations of the ether from methanol yielded ether assaying 14.80 per cent methoxyl; this melted at 54°-55°C the first time and subsequent melting points were 48.7°-49°C. The melting point of the natural (D) isomer was reported as 58°-59°C, but methoxyl values ranged from 14.06 to 14.72 per cent (26).

Our first attempts at forming the ether were with distilled diazomethane and the ester or free acid, but the yields obtained on evaporation to dryness were somewhat above theoretical.

Gas Chromatographic Analysis. To identify the elution peaks, standard known mixtures were run before each analysis period. Retention times, measured from the air peak, varied slightly from run to run. The retention times were plotted against the number of carbon atoms on semilog paper for each run, and the line obtained from the standard run was shifted slightly for maximal overlapping. It was found that the composition of the mixture affected the retention time: a component occurring in relatively high concentration came off more slowly, while a minor component tended to come off a little early.

The semilog plot for the unsaturated normal esters was parallel to that of the saturated esters. Since no standards were available for the unsaturated hydroxy esters, we simply used the plot from the saturated methoxy esters and shifted it to coincide with the major (C_{24}) peak.

The percentage of each major component was calculated from the ratio of its area on the elution curve to the total area for all the eluted peaks. The area of each well-separated peak was assumed to be the peak height times the width at half the peak height (27). The area of the poorly separated peaks was determined by weighing a trace of the entire section. The separation and quantitation of the shorter acids could have been improved by use of a lower temperature and longer column, but these (except in the case of the saturated normal acids) constitute a very small proportion of the acids found.

The precision of the quantitative analyses was estimated by analyzing mixtures of known composition made from palmitate, arachidate, and lignocerate. The lignocerate contained 1.9 per cent behenate; the arachidate, 1.8 per cent stearate. These values were used to correct the weights in the standard mixtures. Analytical results are shown in Table 1. A 2 per cent error is common, but some errors reach 5 per cent. The reported values for the unknowns are derived from three runs, so the largest deviation is probably no more than 2 per cent. Similar tests with standard mixtures of saturated methoxy esters showed similar variability.

Typical curves obtained with the four groups of esters are shown in Figures 1 to 4. The very early peaks from the solvent were omitted from the drawings. The numbers above the peaks identify the num-

[6] By A. Hajra, in this laboratory.

[7] By Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

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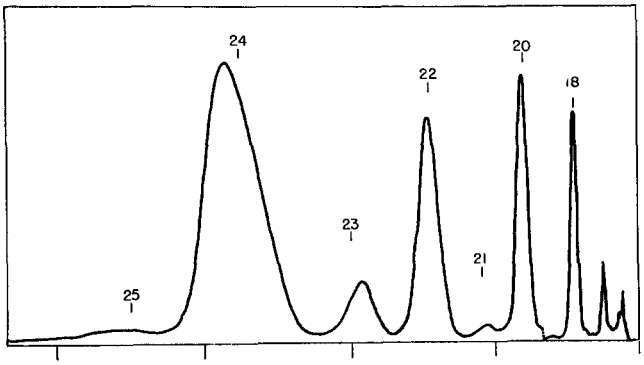

FIG. 1. Gas chromatogram recordings of saturated normal methyl esters obtained from rat brain cerebrosides. The short vertical lines on the abscissa represent 10-minute intervals. The dotted line just after stearate indicates a fourfold increase of sensitivity in the recorder.

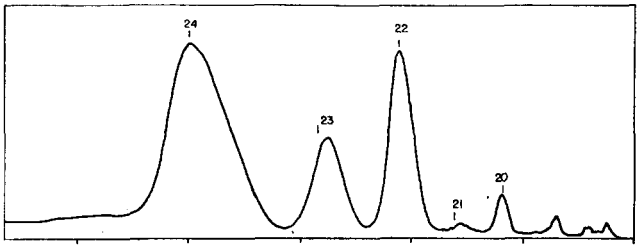

FIG. 2. Gas chromatogram recordings of saturated methoxy methyl esters from rat brain cerebrosides.

ber of carbon atoms in the acid, and the short vertical line just below each number gives the expected peak position.

There is a marked similarity between the two saturated groups and also between the two unsaturated groups. The unsaturated acids contain primarily the known C_{24} compounds, nervonic and α-hydroxynervonic acids, whereas rather large amounts of the C_{23} and C_{22} acids appear in the saturated groups. All four groups contain distinct amounts of the lower homologues, down to C_{16} or less. The shorter acids seem to include isomers, possibly with branched methyl groups, or, in the case of the unsaturated acids, with several double bonds. The similarities in distribution between the hydroxy and normal acids of both types strongly suggest a precursor-product relationship. Further studies on this problem with C^{14}-labeled lignoceric and cerebronic acids are under way.

In the case of the normal acids, it seems likely that part of the lower homologues arises from the impurity known to be present in small amounts in our cerebroside preparations. This explanation is supported by the finding that different preparations yielded somewhat varying contents of the shorter acids. However, Klenk has reported the occurrence of small amounts of unsaturated shorter acids in brain cerebrosides (28, 29). The presence of a longer unsaturated acid, possibly C_{26}, was also reported. Our gas chromatographic column would not separate the Δ^{17} positional isomer of hydroxynervonic acid that was found by Klenk and Faillard (30). We found that there were one or two peaks for acids longer than C_{24} in all four fractions, although the particular curve for unsaturated normal acids chosen for redrawing does not show this. The positions of the peaks do not correspond to straight-chain C_{25} or C_{26} acids, but it is possible that serious shifting occurs with traces of such long acids. In any event, the amounts of longer homologues are very low. The occurrence of behenic acid in a cerebroside from the spleen of a patient with Gaucher's disease has also been reported (31).

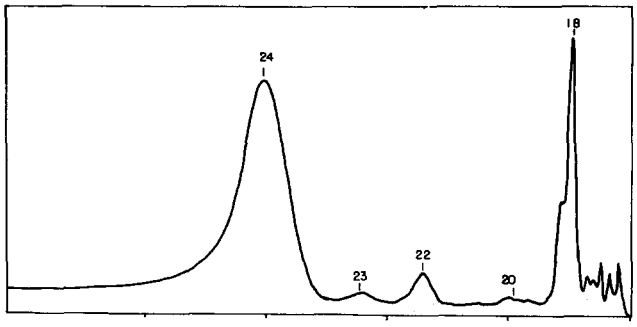

FIG. 3. Gas chromatogram recordings of unsaturated normal methyl esters from rat brain cerebrosides.

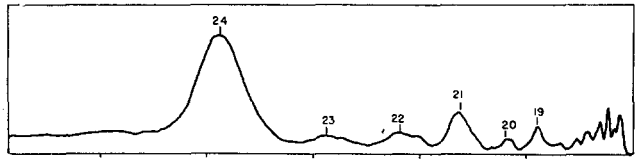

FIG. 4. Gas chromatogram recordings of unsaturated methoxy methyl esters from rat brain cerebrosides.

The existence of C_{22} and C_{26} hydroxy acids, as well as C_{24}, in cerebrosides has been claimed on the basis of X-ray diffraction analyses (32, 33). Wool fat has been shown to contain α-hydroxy acids 10 to 18 carbon atoms long (34) and very recently Skipski *et al.* (35) have found hydroxystearic acid in beef spinal cord cerebrosides. Some of our less pure lipid preparations yielded much higher contents of hydroxypalmitic and hydroxystearic acids, and the origin of these is under study here.

The actual content of each cerebroside acid varies considerably with age; data describing this variation are presented in the article following (36), page 79.

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