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Microdetermination, isolation, and gas-liquid chromatography of 2-hydroxy fatty acids*

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SUMMARY

A new method is described for the specific isolation of 2-hydroxy fatty acids, particularly from brain lipids. The procedure involves saponification of the lipids with KOH-propylene glycol, preliminary purification with a small silica gel column, and precipitation as the copper chelate. The total content of 2-hydroxy acids may be determined by direct colorimetric determination of the copper in the precipitate, or the acids may be liberated and analyzed by gas-liquid chromatography. The acids are methylated with dimethoxypropane and acetylated with isopropenyl acetate prior to gas chromatographic analysis. The method is suitable for samples containing as little as 0.6 μmoles of hydroxy acid, but is readily scaled up for preparative isolations. An improved titrimetric procedure for copper is described.

Fatty acids containing hydroxyl groups at various substitution points occur widely distributed in nature (1). Of particular interest to us are the 2-hydroxy acids, which are quite characteristic of the lipids of the central nervous system. We have used Florisil chromatography followed by gas-liquid chromatography (GLC) to study the 2-hydroxy acids of brain cerebrosides and have found that relatively large quantities of shorter and longer homologs of cerebronic acid (the major acid) occur (2). We also found that the proportions of the odd-numbered acids increase strikingly with age (3), that there is very slow metabolic breakdown of the acids (4), and that they are made by elongation of the shorter fatty acids and degradation of the longer ones[1] (5).

For further study of the 2-hydroxy acids, particularly in crude incubation mixtures, it seemed advisable to develop a more specific isolation method, since the Florisil column could not distinguish between positional isomers or acids containing several polar substituents. Because of their structure, 2-hydroxy acids should form metal chelates whose physical properties might be somewhat different from the salts of other acids that might be present. Klenk used magnesium and lithium chelation, taking advantage of the lower solubility of the chelates (6, 7). Radin et al. (8) found that the copper chelate was very light in color, in contrast to the deep blue copper salts of normal acids, and was somewhat more insoluble. Presumably this pale chelate differs from the purple chelate formed in an alkaline tartrate-copper solution in that the latter contains chelated hydroxyl groups that lack their protons, and the former contains the nonionized hydroxyl group.

Based on the above observations with copper, Rapport et al. (9) proposed a method of estimating the proportion of hydroxy acids in a mixture of acids. At the time the present study was completed, Milburn and Truter (10) reported on the use of copper precipitation for the preparative isolation of 2-hydroxy acids from wool wax.

The method described in this paper begins with a high-temperature saponification, which is needed for liberation of the hydroxy acids from their amide linkage, characteristic of their occurrence in brain. The acids thus obtained contain a polar impurity that is precipitated by copper and thereby causes a falsely high value. This impurity is removed by silica gel chromatography in a single step. Where large amounts

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[1] Hjara, A. K., and N. S. Radin, unpublished experiments.

of normal acids are also present, some of these may also precipitate with copper; they are readily removed with the same silica gel column by an additional elution step. The chelate is then precipitated with copper oleate solution, and the complex is cleaved with a chromogenic reagent, or with HCl-MeOH.[2] In the former case, the copper is determined colorimetrically. In the latter, the copper and acids are separated with water and ether, and the acids are analyzed by GLC. The copper in the aqueous layer may be determined by an improved titration method or colorimetrically.

The method allows one to determine the total amount of 2-hydroxy acids and the amounts of the individual acids in samples containing as little as 0.6 μmoles of a mixture of 2-hydroxy acids. Starting with a brain sample, the entire analysis can be carried out in 2–3 days.

MATERIALS AND METHODS

Materials. Copper oleate solution was prepared by stirring 20 g cupric acetate (neutral) with 20 g oleic acid (technical grade, 93%) in 100 ml chloroform for 2 hr. The filtrate from this mixture was stored in the dark and discarded if a precipitate formed on storage. The copper concentration was 0.124–0.163 M.

The saponification solution, about 1 N KOH, was made by warming 6.5 g of KOH (reagent grade, 85%) in 100 ml of 1,2-propanediol. Stored in the dark, the light yellow solution seemed to be stable at least 3 weeks.

The chromogenic reagent was DDC,[2] 0.1% in *n*-butanol, reagent grade (11). While the data reported here were obtained with this reagent, it was subsequently found that addition of 10% water rendered the reagent more stable and speeded dissolving of the copper compounds.

The standard used for colorimetry was reagent grade cupric chloride, 10^{-4} M in ethanol containing a drop of HCl. Aliquots were evaporated to dryness *in vacuo* in test tubes.

For the titrimetric determination of copper, 0.005 M EDTA[2] was prepared in water. The concentration of reagent based on weight agreed well with that determined by titration against the copper precipitated by pure 18h:0.[2]

[2] Abbreviations used: MeOH, methanol; EtOH, ethanol; DDC, sodium diethyldithiocarbamate; EDTA, disodium ethylenediaminetetraacetate; DEGS, polymer of diethyleneglycol succinate. Fatty acids are identified by their carbon number and number of double bonds (24:0 = lignoceric acid). An *h* indicates the presence of a 2-hydroxyl group (24h:0 = cerebronic acid).

Gifts of various acids are gratefully acknowledged: 2- and 3-hydroxydecanoic;[3] pure 12:0, 16:0, and 18:0[4]; mycolic[5]; 16-hydroxypalmitic.[6] Standard mixtures of normal methyl esters were kindly furnished by the Metabolism Study Section, National Institutes of Health. 2-Hydroxy acids were synthesized from normal acids by the method of Müller (12), and 2-oxopalmitic acid was made from 16h:0 by oxidation with chromic acid (13).

The 95% EtOH[2] (USP), Skellysolve B (hexane), chloroform (USP), MeOH (ACS), 2,2-dimethoxypropane, and isopropenyl acetate[7] were redistilled from a glass still. The silica gel[8] was used as furnished.

Most of the studies reported here were made with a lipid extract of pig brain[9] (mainly white matter), prepared by homogenizing the tissue in 20 volumes of chloroform–MeOH 2:1 (15) and filtering. The solution was stored at −20° and soon yielded a fine precipitate of protein. The precipitate was resuspended before each aliquot was pipetted.

Equipment. The gas chromatograph[10] was equipped with a hydrogen flame ionization detector and a temperature programer. Two columns were used for each sample: a 12-foot column containing Gas-Chrom Z,[11] 80–100 mesh, coated with 1% G.E. silicone SE-30; and a 3-foot column containing the same support, coated with 15% DEGS.[2, 11] The columns were stainless steel, 0.091 in. i.d.

Low-pressure evaporations were performed with a commercial rotary evaporator or with the "Swirler," a high-vacuum evaporator utilizing a rotary pump and a dry-ice cooled condenser (16). The latter is particularly suited to removal of HCl, water, and toluene.

"Vortexing," mixing performed with an eccentrically rotating rubber cup,[12] was used to resuspend solids in liquids and to mix liquid-liquid systems in test tubes.

Thin-layer chromatography was performed with Silica Gel G[13] and the Desaga-Brinkmann equipment.[13] The plates were developed with Skellysolve prior to use and dried 15 min at 105°. The solvent used was Skellysolve B–absolute ether–acetic acid 90:10:1

[3] John H. Law and Norah Johnston, Harvard University.
[4] H. Konishi, Daiichi Kogyo Seiyaku Co.
[5] Hans Noll, University of Pittsburgh.
[6] H. Ageta, Showa Pharmaceutical College.
[7] Gift of Union Carbide and Carbon Corp., New York, N.Y.
[8] Prepared according to Hirsch and Ahrens (14) by Bio-Rad Laboratories, Richmond, Calif.
[9] Gift of Peters Sausage Co., Ann Arbor, Mich.
[10] Model 609, F & M Scientific Corp., Avondale, Penna.
[11] Applied Science Laboratories, Inc., State College, Penna. The Gas-Chrom Z is a siliconed diatomaceous support.
[12] Vortex Jr. Mixer, Scientific Industries, Inc., Springfield, Mass.
[13] Brinkmann Instruments, Inc., Great Neck, N.Y.

(17), or Skellysolve B–absolute ether 85:15 (18). The spots were visualized with alkaline bromothymol blue (19).

Radioactive samples were dissolved in toluene–absolute EtOH–terphenyl–POPOP 950:50:4:0.1 and counted in a liquid scintillation counter.

General Procedure. A solution of brain lipid containing 0.6–2 μmoles hydroxy acid was evaporated to dryness in a 20 x 150-mm screw-cap test tube. A boiling chip and 2 ml saponification solution were added, and the liquid was refluxed 30 min in a 200–210° oil bath kept in a hood. The screw cap was left loose during the first 3 min and then tightened. Part of the saponification solution was left above the level of the bath so that the walls of the tube act as a reflux condenser.

The acids were obtained by adding 3 ml water and 0.3 ml HCl, cooling under ice, and extracting 3 times with 2 ml ether. The extraction was performed in the same test tube by hand agitating and vortexing, and the ether layers were transferred to another screw-cap test tube with a Pasteur pipette. The pooled ether extracts were washed by vortexing with 2.5 ml of 4% KCl and 3 ml of toluene–EtOH 5:1, then centrifuging and drawing off the supernatant solution. The toluene served to remove most of the water from the ether, obviating the need for desiccation by sodium sulfate. The toluene–ether solution was evaporated to dryness in a 50-ml flask using a rotary evaporator, and a second evaporation with 2 ml benzene was performed to remove remaining water.

The hydroxy acids were next purified by chromatography. A column of 6 mm o.d. tubing was prepared with glass wool and sand on the bottom. A stopcock was unnecessary. One-half gram of silica gel was slurried with 1.5 ml benzene with the aid of an ultrasonic bath, then pipetted into the column. The transfer was completed with the aid of 3 ml benzene, a little sand was dropped onto the top of the packing, and 5 ml more benzene was passed through for conditioning. The sample of fatty acids was then dissolved in 1 ml benzene and applied to the packing. An additional 4 ml benzene was used to complete the transfer and to elute the normal fatty acids. Next, the hydroxy acids were eluted with 7 ml of benzene–absolute ether 9:1. Compressed air at 2 psi was applied to the top of the column throughout the chromatography, yielding a flow rate of 25–30 ml/hr.

The hydroxy acid fraction was evaporated to dryness with a rotary evaporator, then transferred to a 2-ml conical centrifuge tube with small portions of ether. The ether was removed with a stream of nitrogen. To bring the acids to the bottom of the test tube, the walls were rinsed down with a few drops of ether and the evaporation was repeated.

The tan residue was dissolved in 0.06 ml of absolute EtOH by brief warming, making sure that the EtOH vapors washed down any acid that might be on the walls. Shortly after cooling, 0.02 ml of cupric oleate solution was added and the mixture was covered with tinfoil and left for 10 min in a refrigerator. Precipitation of the chelate was completed by storing an additional 20 min at room temperature.

The supernatant solution was removed by brief centrifugation and decantation, and the pale blue precipitate was washed twice with the same solvent, absolute EtOH–chloroform 3:1. The washing was done by rinsing down the test tube walls with 3 drops, vortexing, rinsing down the walls with 3 more drops, and centrifuging.

The chelate was dissolved by warming 2 min at 60–65° with 0.5 ml of MeOH–concentrated HCl 9:1. The cooled solution was transferred to a 25 x 200-mm screw-cap test tube with the use of 5 ml ether as a rinse. Five milliliters each of toluene and water were added and the mixture was vortexed and centrifuged. The supernatant fluid was used for isolation, or GLC, of the hydroxy acids, and the aqueous layer was used for determination of copper.

Copper Determination. In the spectrophotometric method, the aqueous solution is evaporated to dryness in the same test tube with the swirler. The DDC solution is added, vortexed, and the absorbancy of the resultant solution measured at 435 mμ. Ten milliliters of DDC solution are used for 1 μmole hydroxy acid; 20 ml for 2 μmoles. The blank is made by mixing MeOH, HCl, ether, toluene, and water, and treating just as with the chelate. The content of copper is determined by comparison with a copper standard. One μmole of copper corresponds to 2 μmoles of hydroxy acid.

If recovery of the hydroxy acids is not desired, the DDC solution should be added directly to the chelate. The small size of the test tube necessitates mixing by an indirect procedure. Ten or 20 milliliters of DDC are pipetted into a test tube and a portion of this is transferred to the precipitate with a Pasteur pipette, which is also used to suspend and dissolve the precipitate. The liquid is returned to the test tube, mixed, and another portion transferred to the 2-ml tube. This transferring and mixing is done a total of four times, after which the absorbancy is measured.

One-half μmole of copper gives an absorbancy of 0.66 when measured in a 1-cm path cuvette in a Beckman DU spectrophotometer.

Analysis of the Hydroxy Acids. The ether–toluene solution contains a mixture of free acid and methyl ester. The free acid was esterified by evaporating the solution to dryness in a 50-ml flask and adding 1 ml of freshly mixed MeOH–concentrated HCl–dimethoxypropane 10:1:25. Use of this mixture is more convenient than separate pipetting of the individual liquids (20) and is equally effective, as shown by Florisil chromatography[1] and thin-layer chromatography. The flask was stoppered and left for 60 min; after the addition of 5 ml of toluene, the solution was evaporated to dryness with the swirler.

The esters were transferred with ether to a conical test tube made from a 13 x 100-mm screw-cap test tube. As with the copper precipitation, nitrogen and a second evaporation are used to bring the sample to the bottom of the tube. In our earlier work, we methylated the hydroxyl groups to make the hydroxy esters more volatile (2), but this seemed a little difficult to do with 0.6 μmole. Instead, we acetylated with 50 μl of isopropenyl acetate–sulfuric acid 500:1, freshly mixed (21). The tube was sealed with a Teflon-lined screw cap and the lower tip was heated in a 60° bath for 30 min. The yellow solution was mixed with 0.3 ml water and 0.3 ml of ether–toluene 1:1, then vortexed, and centrifuged briefly. The upper layer was transferred with the aid of a small amount of ether to a small screw-cap test tube. To remove traces of water and acid, several grains of potassium carbonate were shaken with the solution and left for 15 min. The solution was transferred and evaporated, with ether rinses, into the tip of another conical screw-cap test tube.

The residue of esters was dissolved in carbon disulfide to yield a concentration of 25 μg/μl. For chromatography on the SE-30 column, 1–2 μl were injected for each run; for the DEGS column, 5 μl. The larger sample for the DEGS is a consequence of the greater bleeding shown by this packing; use of more esters allows use of a lower sensitivity and the effect of bleeding on the base line is thereby diminished.

Alternative Copper Determination. A titrimetric method for analyzing the chelate was used in some of the experiments. Here, the chelate was cleaved by warming with 0.5 ml of N sulfuric acid in MeOH and the solution was transferred to a screw-cap test tube with 5 ml each of ether, water, and toluene. The aqueous layer was transferred to a 25-ml flask with 2 rinses of 1 ml each. Two drops of 0.1% ethanolic thymol blue were added and the solution, under magnetic stirring, was brought to a pale pink (pH 2.8) with 0.3 N ammonium hydroxide. The solution was next heated to near boiling and immediately titrated with EDTA to the colorless point. The indicator consisted of 2 drops of an ethanolic solution containing 0.1% 1-(2-pyridylazo)-2-naphthol (22) and 0.1% Brilliant Cresyl Blue; the initial color was violet. Occasional reheating of the solution may be necessary during the titration. A microburet was needed as 1 μmole of hydroxy acid chelates 0.5 μmole of copper, which corresponds to 0.5 μmole or 0.1 ml of EDTA.

The use of Thymol Blue and Brilliant Cresyl Blue constitutes an improvement over the previously described titration method (23), as the pH indicator gives the close control necessary to prevent indicator precipitation and the dye greatly sharpens the color change.

If recovery of the hydroxy acids is unnecessary, one can titrate the entire mixture directly. The MeOH–sulfuric acid solution of the chelate is transferred with 5 ml of water to the titration flask. Although the water precipitates the lipid, one can still readily follow the color changes.

TABLE 1. DETERMINATION OF 2-HYDROXY ACIDS IN DIFFERENT AMOUNTS OF BRAIN LIPIDS

Volume of Extract*	Addition of Normal Esters†	Hydroxy Acid Content	
ml		*μmoles*	*μmoles/ml extract*
0.912	5-fold	0.578	
		.594	0.636
		.579	
1.60	none	.986	
		.976	0.618
		1.004	
1.60	5-fold	1.012	
		0.996	0.629
		1.015	
3.20	5-fold	2.024	
		2.018	0.629
		2.000	

* 1 ml of extract corresponds to about 48 mg of pig brain.

† The normal esters added were those present in the same extract, isolated by the Florisil procedure, and added back in proportion to the amount already present in the sample.

RESULTS AND DISCUSSION

Reproducibility. The difference between duplicates or triplicates carried through the entire procedure was usually less than 3%, using the colorimetric or titrimetric methods (Table 1). Since the copper standards agreed much better than this, the variability is derived from the preceding steps.

The reproducibility of the GLC analyses, in which only percentage composition measurements were made, varied with the column, type of mixture, and percentage of the individual component. A National Institutes of Health standard mixture of normal esters gave values agreeing within 0.3% of theoretical. More complex mixtures of normal and acetoxy esters, 15:0 up to 26h:0, were compared on our thermal conductivity gas chromatograph.[1] Here the deviations were greater, going as high as 2% absolute for a 25% component, 1.4% for a 12% component, and 0.37% for a 0.77% component. The variability with the ionization gas chromatograph was smaller than the deviations.

Our SE-30 column separated the saturated from the unsaturated esters only slightly, just enough to show the presence of a mixture. However, separation of homologs differing by one carbon atom is readily accomplished. With the DEGS column, separation of 24h:0 from 24h:1 is almost complete, but the 24h:1 peak overlaps the 25h:0 peak to a significant extent. Similarly, there is some overlap between 25h:1 and 26h:0. Since the odd-numbered hydroxy acids occur at unusually high levels in most samples thus far examined, the precision of such analyses suffers. We routinely use both columns for each sample and use the data from each to correct for the contents of the unsaturated homologs.

Branched chain acids are reported to appear just before the corresponding normal acids with *both* columns (24, 25). There was no indication in any of our elution curves of the presence of such peaks. Moreover, the area of each compound peak (from SE-30) was almost equal to the sum of the two areas calculated from the corresponding saturated and following unsaturated peaks (from DEGS).

Temperature programs of 1.3°/min were used, and the range was 170–205° for the SE-30 column and 150–200° for the DEGS.

Linearity of the Method. The titrimetric and colorimetric copper procedures gave readings that were proportional to the weight of copper used. Linearity was also demonstrated with a variety of 2-hydroxy acids, processed through the chelation and copper determination. Good results were obtained with acids 10 to 24 carbon atoms long, with sample sizes up to 47 μmoles and suitably adjusted solution volumes.

Another linearity test was made of the entire procedure, starting with brain lipids and using the extraction-colorimetric method. This experiment was combined with an interference study, described later. Table 1 shows satisfactory linearity between 0.58 and 2.01 μmoles. An attempt to use a smaller sample, containing 0.22 μmoles, gave some low values.

Recoveries and Absolute Values. The same brain extract was analyzed by a modification that combined a previous method (5) and the chelate method. The lipid from 200 ml of the extract was cleaved with MeOH–concentrated HCl, the nonsaponifiables were removed, the acids were converted to esters, and the polar ester fraction was obtained by Florisil chromatography. This fraction was saponified under mild conditions, with 1.9 ml 95% EtOH and 0.1 ml of 40% KOH at 80° for 2 hrs, and the resultant acids were analyzed by the copper colorimetric procedure. The hydroxy acid content was 105.4 μmoles, corresponding to 0.527 μmoles/ml of extract. This is 15% lower than the value of 0.618 found as the average of many analyses run by the short method on this batch of lipid.

The methyl esters isolated from the chelate from the long procedure weighed 41.6 mg. Gas chromatography showed an average molecular weight for these acids of 390; this weight corresponds to 0.533 μmoles/ml of extract, 14% lower than the value obtained by the short copper method. Gas chromatography of the esters obtained by both methods showed slight differences, particularly in the shorter acids, and it may be that the losses in the early stages of the long method are greater for the longer acids. The shape of the peaks were similar, suggesting that the high temperature saponification and short procedure did not introduce artifactitious esters.

Another type of comparison was made with a sample of pure cerebrosides (26), which ordinarily constitute the major form of bound hydroxy acids in brain. Here, only the older method (2) was used and the hydroxy esters obtained from the Florisil column were simply weighed. The average of several analyses gave a value of 33.9% of the cerebrosides. The new method, using copper titration, yielded a value of 0.868 μmoles, or 33.9% by weight.

A recovery test was made in duplicate by adding 3.05 mg (2.64 μmoles) of the same cerebroside sample to a portion of lipid containing 6.18 μmoles of hydroxy acid. The final value, from the titration method, was 8.75 μmoles, corresponding to a recovery of 97.5%.

One would expect that the chelates of unsaturated hydroxy acids would be more soluble than those of the saturated ones, and there might be low recovery of the former. The agreement found in the above experiment with pure cerebrosides shows there is no loss of unsaturated hydroxy acids. As a final check, we analyzed the supernatant solution from a copper precipitate by thin-layer chromatography and found no 2-hydroxy acids.

Interference Tests. The first question considered was the possibility that normal acids (primarily the

saturated ones) might precipitate as the copper salts with the chelate. Copper oleate was chosen as the precipitating agent partly because of its solubility in lipid solvents, and partly because it was felt that the excess oleic acid and acetic acid (derived by the mode of preparation) would tend to prevent precipitation of the normal acids.

Tests were made with hydroxystearic acid containing varying amounts of stearic acid and it was found that high copper values were obtained when the 18:0/18h:0 ratio was 7 or more. At this point, a noticeable deepening of color was evident in the otherwise pale blue precipitate.

In the case of typical brain total lipids, the ratio of normal saturated acids to hydroxy acids is not far different from 7, so attempts were made to precipitate the chelate directly from the mixed fatty acids. However, the precipitate formed in erratic yield and had a brownish color. Evidently the crude lipids, on high-temperature saponification, yield some material(s) that inhibit precipitation, and coprecipitate as copper compounds. Pure cerebrosides, on the other hand, can be saponified, and the fatty acids treated directly with the cupric oleate.

Silica gel chromatography was investigated for removal of the interfering material and the normal fatty acids. Trials with labeled methyl stearate added to brain lipids showed that the normal acids and cholesterol could be eluted with 15 ml of benzene (from a 0.5-g silica gel column), and the hydroxy acids with 5 ml of benzene–ether 9:1. Thin-layer chromatography of effluent fractions showed the presence only of fatty acid and cholesterol in the first solvent, but the polar effluent contained some slow-moving substances. It was also shown that our standard procedure, in which only 5 ml of benzene is used in the first step, elutes much of the normal acids but leaves a good deal of the cholesterol for elution with the hydroxy acids. Apparently the copper-precipitating substance is not eluted under these conditions, so a sample that is not too high in normal acid content could be analyzed by omitting the benzene elution. The volumes of solvents needed in the chromatographic step should, of course, be checked for each new batch of adsorbent.

It may be presumed that the loading capacity of a given column is almost independent of the weight of the first fraction to be eluted, provided the entire sample is added in the eluting solvent. A check on this point is shown in Table 1, derived from an experiment in which the amount of total lipid was varied and the amount of normal acids was raised to 5 times the natural level (normal/hydroxy = 45.6). The values seem independent of the addition and sample level, within experimental error. Thus, use of the silica gel column and two eluting solvents enables one to analyze lipids that are extremely low in their content of 2-hydroxy acids.

In recovery tests with pure 2-hydroxy acids from beef spinal cord, obtained by the Florisil method, it was found that approximately 0.05 μmoles remained on the silica gel column even though the amount applied to the column varied. All the analytical data reported here were corrected for this loss. Perhaps the slightly higher values shown in Table 1 for the heavily loaded columns are the result of slightly diminished adsorptive loss. Use of a wider column with the same weight of silica gel gave erratic data, indicating that the interfering material had escaped from the column.

A variety of lipids was examined for precipitability in the standard chelation step. No precipitate was obtained with cholesterol (2.5 μmoles), stearyl alcohol (3.1 μmoles), 16-hydroxypalmitic acid (2.7 μmoles), ricinoleic acid (1.1 μmoles), 2-oxopalmitic acid (2.7 μmoles), and 3-hydroxydecanoic acid (0.97 μmoles). The last two acids were particularly interesting since it seemed possible that they would form chelates under these conditions. Mycolic acid (1.26 mg), another 3-hydroxy acid, did form a precipitate, but the precipitate was blue, showing that this merely forms a very insoluble copper salt. The molecular weight of this acid is very high and it is very insoluble even in EtOH–chloroform at room temperature. The inactivity of cholesterol is convenient since this allows us to save time in the benzene elution step.

Incidentally, it should be pointed out that the copper chelate method furnishes additional evidence to confirm the structural identification of the rarer 2-hydroxy acids of brain, particularly the 23h:0, 25h:0, 26h:0, 25h:1, and 26h:1 (3, 27).

It should be possible to analyze whole brain without bothering to extract the lipids, and a preliminary test was made with 100 mg of proteinaceous residue obtained from lipid extraction. The residue was saponified, acidified, and extracted with ether. The residue from the ether, before or after silica gel chromatography, did not give a precipitate with copper. From this we conclude that no artifacts are produced by the treatments and, incidentally, that there can be very little bound hydroxy acid left unextracted in the Folch procedure.

A series of experiments made on lyophilized aliquots of homogenized whole brain yielded promising results, but replication was poor. Presumably the variation was due to the difficulty of getting typical aliquots from the pasty mass. Little improvement was obtained when we pipetted aliquots of whole brain

homogenized in water and lyophilized the suspensions in the saponification tubes. Perhaps the best way of testing the applicability of the method would be to analyze two large groups of similar rat brains by the two methods: whole brain vs extract of whole brain.

Study of the Saponification Conditions. A survey of the literature on hydrolysis of cerebrosides with alkali disclosed that this method has received singularly little investigation. In the saponification of cerebrosides to yield psychosine (28), the lipids (which reside mainly on the upper walls of the reflux flask) are heated 10 hr with barium hydroxide in water–dioxane. It seemed likely that the lack of use of alkali was due to failure to consider the solvent properties of the glycolipids and the fact that an amide linkage is relatively stable. We accordingly investigated the use of a stronger alkali (KOH) in a nonaqueous solvent (propylene glycol), which has a high reflux temperature (about 190°). Such a system was described for the analysis of amides with hydroxylamine (29).

Table 2 shows the results of a study using brain lipids with two concentrations of KOH and varying refluxing periods. The data indicate that 2 N KOH and 1 N KOH yield similar results, and that the hydroxy acids are completely liberated in 20 min. Thirty minutes was chosen as a safe minimum period. There may be a tendency for increased heating time to yield slightly more hydroxy acids. If this is real, the increase might be due to a slow reaction producing an artifactitious copper-precipitating compound; all the more reason for keeping the reflux period short. A pleasant feature of this method of cleaving lipoidal amides is the marked reduction in reflux times as compared with previously published methods.

TABLE 2. EFFECT OF CONCENTRATION OF KOH AND OF SAPONIFICATION PERIOD

Concentration of KOH in Propylene Glycol	Duration of Refluxing	2-Hydroxy Acid Content*
N	*min*	*μmoles/ml extract*
1	10	0.554
1	20	0.593
1	30	0.604
2	30	0.592
1	60	0.608
2	60	0.606
1	120	0.620

* Average of 2 or 3 values.

The main disadvantage of the KOH-propylene glycol system is that it causes migration of the double bonds of polyunsaturated acids. At present, there is no report of polyunsaturation in the 2-hydroxy acids or in the glycolipids. Comparison of the gas chromatograms of the hydroxy acids obtained by the KOH and HCl methods of splitting suggest the amount of such acids must be small.

It would seem advantageous to use lipid samples that are free of water, as this might lower the boiling point of the solution. We evaporated our extracts, which contained about 4% water, with nitrogen in a 60° bath and completed the drying in a vacuum desiccator over KOH for at least 30 min. We also evaporated samples under vacuum with the swirler, adding toluene to prevent foaming. A second vacuum evaporation was made after adding toluene–MeOH 1:1.

The Precipitation Step. The chelate of 18h:0 was prepared on a large scale and its properties were studied. The compound is very insoluble in a variety of solvents and could not be recrystallized. It has no clear melting point but gradually turns brown above 210°. The analytical data suggest that the chelate is a half-hydrate:

$$(C_{18}H_{35}O_3)_2Cu \cdot 1/2\ H_2O.$$

Calculated: C 64.40, H 10.68; found: C 64.37, H 10.47

The water of hydration may come from the cupric acetate (monohydrate) used in making the oleate and from the traces ordinarily found in commercial absolute EtOH.

In preliminary studies of the ratio of EtOH to copper reagent, it was found that a ratio greater than 3 tends to precipitate the normal acids, and a ratio less than 3 makes centrifugation difficult because of the density of the solution. Tests with 18h:0 showed that precipitation may be incomplete when its concentration is less than 2 μmoles/ml of final solution. Washing the precipitate with EtOH–chloroform 3:1 does produce some loss, less than 0.05 μmoles/ml wash solution.

In applying this precipitation to a novel mixture of lipids, one should keep in mind the possibility that the solvent conditions used here may not dissolve the non-2-hydroxy acids or their copper salts. In the former case, a test run should be made with EtOH and chloroform. If no precipitate forms, it is safe to evaporate to dryness and proceed with the copper reagent. If a precipitate does form, this might be an insoluble 2-hydroxy acid and one should proceed as above anyway, to see if the precipitate is deep blue or pale blue. Obviously it is wise to investigate the matter further with thin-layer chromatography.

The Colorimetric Step. The colored compound is quite stable, possibly increasing in intensity by 1% in 20 hr. Comparison of cupric chloride and acetate solutions by titrations with EDTA showed that these copper standards contained the amounts expected from the weights. The blank absorbancy, compared with water, was 0.005–0.010 in a 1-cm cuvette.

Preparation of the Fatty Acids for Gas Chromatography. Attempts to form the methyl esters directly from the chelate by heating with MeOH–sulfuric acid gave at best a yield of 85%. When the chelate was decomposed with MeOH-HCl and dimethoxypropane was added directly to the solution, the conversion was complete but the weight yield was somewhat greater than theoretical. Thus the final procedure calls for removal of the copper before completing the esterification.

Comparisons made on a larger scale showed that the copper content of the chelates obtained from brain lipids and from cerebrosides was that expected from the weight of the precipitate.

The acetylation method, based on the findings of Hagemeyer and Hull (21), appears to be virtually quantitative. Thin-layer chromatography showed only a trace spot for the nonacetylated compound. The R_f values in the 90:10:1 solvent described earlier were: methyl 18:0, 0.86; methyl 18h:0, 0.24; 2-acetoxy methyl esters of spinal cord, 0.43. Florisil chromatography of the acetoxy compound gave a yield of over 97%, the derivative being eluted with Skellysolve B–absolute ether 99:1.[1]

Application. While the experiments described here were performed mainly on lipids of the central nervous system, the procedures in their various forms are applicable to other lipids. The accompanying paper (30) describes a study of the occurrence of 2-hydroxy acids in rat tissues. The basic method is suitable for duplicate analysis of samples as small as 40 mg of cerebral white matter or 250 mg of cerebral gray matter. The method is also suitable for determining the molecular weight of 2-hydroxy acids on a 0.6 μmole scale.

In the basic method, part of the normal acids are allowed to come off the column with the hydroxy acids so that analysis of the normal acids in the benzene eluate would give low values. However, the total normal acid content of a sample could be determined simply by raising the volume of benzene. The normal acid content of this fraction could be determined by titration or the copper extraction method of Duncombe (31).

Parts of the regular procedure can be omitted according to the requirements of the problem. Low-temperature saponification can be used where the hydroxy acids are ester-linked, as in wool fat. The silica gel column is not always needed, as in cerebroside analysis, or it can be used with only a single solvent elution step, as in lipid samples high in cerebroside content. The scale of work, especially the precipitation step, can be readily increased for preparative isolations. With the aid of the procedure, we have succeeded in demonstrating the synthesis of 2-hydroxy acids *in vitro* by brain preparations, starting with acetate-C^{14}.[1]

Because of the widespread occurrence of distinct amounts of polar acids, it would seem wise for those working with the normal acids to purify the latter by Florisil or silica gel chromatography.

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