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The Hydroxy Fatty Acids: Isolation, Structure
Determination, Quantitation

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Abstract

This review and commentary on quantitative methodology of the hydroxy fatty acids covers methods of isolation, structure determination, and quantitative analysis. Individual topics are: methods of liberation from bound form, separation by partition, separation by partition columns, separation by precipitation, separation by adsorption columns, separation by thin-layer chromatography, separation of homologous fatty acids, locating the hydroxyl group, characterization of the optically active hydroxyl group, locating the double bonds, gravimetric determination, colorimetric determination, gas chromatographic determination, infrared absorption, titrimetric determination, and radiometric determination.

THIS GROUP OF COMPOUNDS has been reviewed fairly recently in articles by Downing (23) and Markley (73), who are to be commended particularly for their compilations of naturally occurring hydroxy fatty acids. The following review will emphasize the problems of analyzing these lipids, and will cover methods of isolation, structure analysis, and quantitative determination. It is assumed that the reader is familiar

with these matters as they apply to ordinary fatty acids, and reference will be made to techniques only if they are relatively unfamiliar or if there is some question whether they can be used with hydroxy acids in unmodified form.

The abbreviations used here are: A, alcohol (ethanol); B, benzene; C, chloroform; E, ethyl ether; H, hexane; M, methanol; W, water; *nfa*, nonhydroxy (ordinary) fatty acids; and *hfa*, hydroxy fatty acids. Numerical designations for *fa* show the number of carbon atoms and the number of double bonds. Thus 18:0 is stearic acid, 18:1 is octadecenoic acid. An *h* is used to indicate a *hfa*: *h*18:0 is hydroxystearic acid. The position of the OH group, in analogy to the common system for showing the position of double bonds, is indicated by a superscript: *h*³16:0 is 3-hydroxypalmitic acid, *h*⁴18:1⁷ is 4-hydroxy-7-octadecenoic acid.

Methods of Isolation

Liberation from Bound Form

Since the *hfa* normally occur in complex lipids, as esters or amides, it is generally necessary to release them from their linkages by saponification or solvolysis. There is probably no complicating difference in ease of splitting such bonds, as compared with the

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ordinary fa. However, almost none of the published procedures for such cleavages have been adequately examined. For any procedure it should be shown, with TLC at least, that no more of the original or a likely intermediate exists in the reaction mixture and that side products are not formed. Thus, in solvolysis (usually in M), one should look for formation of free fa. Since extraction procedures usually follow cleavage reactions, these should be tested for completeness of recovery, most easily with a radioactive fa.

The hfa often occur in amide linkage, attached to the nitrogen of sphingosine or its analogs. Since amides are particularly difficult to cleave, it is particularly important to question the adequacy of the cleavage conditions. Weighing the crude esters is certainly not a safe method of evaluation since sphingosine salts tend to accompany the hfa. For many years Klenk used Thierfelder's method: reflux 4 hr with 10% sulfuric acid (by weight) in M. This method apparently yielded a mixture of methyl esters and free hfa, presumably due to water-forming side reactions. Methanolysis with dry HCl in M has become more popular, probably because free fa do not contaminate the esters. We used to run this step under reflux conditions, but some loss of HCl takes place, and one may assume that the loss will be variable. If the reaction is run in a sealed glass tube, this is no problem.

We have recently taken advantage of the resistance of Viton A, a rubbery halocarbon plastic, to make the methanolysis more convenient (64). A methanolysis tube is made by sealing a heavy wall test tube to an O-ring joint, and a cap is made by simply sealing off another O-ring joint. The two parts are held together, with the Viton O-ring as seal, with a suitable ball-and-socket clamp. The parts are available from Delmar Scientific Laboratories, Maywood, Ill. While we use the flat type of joint, the ball type should work as well; possibly the more common Buna N O-rings would also be suitable. For use, the sample is added to the methanolysis tube, a solution of dry HCl (5%) in M is added, and the cap is clamped on. The methanolysis is carried out at 75C overnight. Other workers have used higher temps and shorter times, such as 100C for 4 hr. Cleavage of esters is, of course, somewhat faster.

It should be pointed out that heating or storing dry HCl in M leads to formation of methyl chloride plus water (11). Since storage for some time does not seem to spoil this reagent, it would appear that the presence of water is not as inhibitory as is generally believed, nor is the concn of the HCl at the time of use very critical. Sweeley and Moscatelli (111) have indeed started with aqueous HCl: they refluxed their samples 5-6 hr with concd HCl-M (1:5). Apparently methyl esters were formed. This hydrolysis mixture, because of its high water content, is not good lipid solvent.

Other workers have used aqueous HCl to cleave the sphingolipids, which means that very little of the sample is dissolved by the medium. Complete release of hfa and nfa was found after 2 hr with 2 N HCl at 110C in a sealed tube (90). Possibly larger samples would not be completely cleaved because the sample and acid phases are immiscible.

Alkaline cleavage of sphingolipids requires high temps. This can be accomplished by refluxing the sample in a screw cap test tube with KOH in propylene glycol (6.5 g and 100 ml) for 30 min (61). This is the fastest method of releasing the hfa, but

polyunsaturated acids might show rearrangements. Saponification has the obvious advantage over solvolysis that nonsaponifiables can be removed by solvent partition; this is particularly useful in large scale work. Acidic cleavage converts 1-alkenyl ethers, if present, into acetals or aldehydes, which are inconvenient to remove from nfa. Probably these lipids are stable to alkaline cleavage and might give less interference.

The hfa require extra precautions, as compared to the nfa, in processing after cleavage. One problem arises from their low solubility in H or petroleum ether. If a saponification mixture in A or M is acidified with HCl and extracted with H, enough of the polar solvent enters the upper phase to take the hfa into the H. However, when the upper phase is washed with W to remove HCl, the W also extracts the polar solvent and the free hfa tends to precipitate out and form an unpleasant gel. For this reason, one should wash the H extract with W-E, W-M, or W-A or else simply use W plus E for the initial extraction. We use the latter in the propylene glycol saponification, and backwash with A-toluene-KCl (61). The KCl prevents emulsions and the toluene squeezes water out of the E extract, making drying less of a problem.

The second problem with hfa arises from the tendency of the hydroxyl group to react with the carboxyl group of the same or adjacent molecules, to form lactides or lactones. Just how serious this problem actually is has not been well determined. In the case of the 2-hydroxy acids, lactide formation is not a problem for attempts at direct synthesis have given low yields (75). It would seem wise to cool the alkaline saponification mixture while acidifying. Another approach is to precipitate the calcium salts by adding calcium chloride to the saponification mixture. The calcium salts are then converted to esters by refluxing the salts with an alcohol and acid (24,22). Theoretically, precipitation of the calcium salts allows removal of the nonsaponifiable fraction, but coprecipitation seems a likely danger and one should extract with H before adding the calcium chloride.

A third problem occurs with hfa in which there is a double bond adjacent to the hydroxyl group (in the allylic position). Such hfa readily dehydrate on heating, and acidic conditions of cleavage or esterification either produce dehydration or ether formation. Satisfactory methanolysis can be achieved by passing the glyceride esters in M through a strongly basic ion exchange column (19). The resin catalyzes the transesterification, albeit fairly slowly. It has the advantage over ordinary alkaline catalysis that there is no problem of catalyst removal.

Separation of Hydroxy Fatty Acids by Partition

Chromatography is the method of choice, but for large-scale work it might be well to try solvent partition methods. Countercurrent distribution has been used for hfa from wool fat (45,88), plant cuticle (74), and fungi (8). Diamond et al. (19) have recently reported the use of this method for purification of methyl dimorphecolate ($h^{9}18:2^{10,12}$), prepared from a seed oil. The distribution was carried out in separatory funnels with acetonitrile and pentane as the partitioning phases. Over 90% of the ester was recovered in a purity of 92%. The actual yield, 57 g, shows the advantage over ordinary scale chromatographic methods.

Noble et al. (88) used heptane and A-M-W as the partitioning phase. Morris et al. (80) found H and

A-W 4:1 to be rather effective, more so than hexane/ acetonitrile. Furfural-H and nitromethane-H have been suggested for such separations (69).

To increase the difference in polarity between nfa and hfa, these workers have used the methyl esters rather than the free acids. Countercurrent methods tend to be sensitive to chain length, as well as the presence of the hydroxyl group, and one tends to get overlapping between short nfa and long hfa.

One can increase the difference in polarity between nfa and hfa by forming a more polar derivative of the hydroxyl group. For example, chlorosulfonic acid reacts quantitatively with hydroxy compounds in anhydrous E, forming HCl and a sulfuric acid ester (7). The sulfate ester should be relatively easy to extract with a suitable alkaline water-containing solvent.

A similar derivative of the hydroxyl group is the half ester of phthalic acid (44) or succinic acid (109), formed by heating the acid anhydride and toluene with the alcohol. The resultant carboxylic acid can then be extracted into an alkaline aqueous solvent. Unfortunately for workers with long-chain hfa, such acidic derivatives form soaps in alkali and give rise to emulsion problems. However, this approach seems worth more trials, with various solvent systems and lower pH's.

Separation of the Hydroxy Acids by Partition Columns

An obvious extension of liquid/liquid countercurrent systems is the partition column, in which one liquid is rendered stationary by suitable spongy particles. Such a separation was made of the methyl esters of seed fatty acids (28), in which the stationary phase—M-B 20:80—was immobilized with silica gel, and the esters were eluted with M-B 2:98. The nfa were well separated from the hfa, and the dihydroxy acids were eluted last, but with some trailing.

A reversed phase partition column has been described for the methyl esters of another seed oil (27). Iso-octane was the stationary phase, supported by siliconized Celite, and the moving phase was acetonitrile. Elution yielded well-separated samples of hfa, epoxy fa, and nfa. The nfa seemed to be separated into saturated and unsaturated fractions.

Such columns can also accomplish some separation according to carbon number, the resolution being suitable usually for fatty acids differing by two carbon atoms. In the case of the hfa, the interaction between hydroxyl groups interferes and it is necessary to derivatize them. The acetate esters of long chain hfa methyl esters were separated with a column in which the immobile phase, mineral oil, was held by siliconized Celite, and the individual hfa were eluted with waer-acetone mixtures (31).

An interesting study of the partition column separation of nfa and hfa describes the use of W-acetone vs. castor oil (38a).

The main advantage of partition columns is that they tend to allow use of relatively large samples. Reversed phase columns are of particular interest because the *more* polar lipids come out first. This may help yield purer preparations of hfa, since a slight amt of trailing can always be expected with any column. The disadvantages are several: many otherwise valuable solvent systems cannot be immobilized satisfactorily; gradient or stepwise elution systems are limited due to the tendency to strip the stationary phase as elution progresses; partition systems tend to be temp-sensitive and the column, as well as equilibrated solvent, may require jacketing;

a number of immobilizing materials are difficult to prepare and use. I believe workers in this field are overlooking a potentially useful immobilizing material, polystyrene beads, slightly crosslinked (97). These are the beads used to make Dowex 50, before the sulfonation (Dow Chemical Co., Midland, Mich.).

Separation of the Hydroxy Fatty Acids by Precipitation

In the special case of the h²fa, chelates can be formed which are markedly more insoluble than the salts of accompanying nfa. Free acids are used for this separation since the esters do not form chelates. Lithium and magnesium chelates have been formed by adding the metal acetate in A to a hot ethanolic solution of the mixed fa (66). The copper chelate is a particularly suitable derivative which can be formed by adding cupric nitrate in A to a solution of the fa in C (75) or by adding cupric oleate in C to a solution of the fa in absolute A (61).

The copper precipitation, under the latter conditions, is highly specific for hfa having the hydroxyl group in the 2-position and will give quantitative yields with as little as 0.6 μ moles. However, large amts of nfa, if present, will also yield some precipitate, as will certain highly insoluble contaminants. Still, the method is very useful for large scale isolations and yields a preparation which can be purified further by reprecipitation or other means. On a small scale, it is very simple to remove excessive amts of nfa by column chromatography and then purify the polar fraction by the copper precipitation. We have found the chromatographic step necessary when working with the complex lipids of brain, not because of nfa interference but because of the presence of some unknown interfering substance (61).

The hfa are recovered from the precipitate by brief warming with coned HCl-M 1:9 and diluting with W-E, or by stirring for some time with aqueous HCl and E. In the former method, one obtains mostly methyl ester; we treat this with coned HCl-M-dimethoxypropane for 1 hr to convert residual acid to methyl ester (96).

Separation of Hydroxy Fatty Acids by Adsorption Column Chromatography

Adsorption chromatography readily yields separation of nfa from hfa. If the free acids are used, they may be separated with a silica gel (silicic acid) column. The nfa (and cholesterol) are eluted with B and the hfa with B-E 9:1 (61). With some batches of silica gel we have found it necessary to add a little E to the first solvent. Preiss and Bloch (95) have performed the separation with pentane-E 85:15 for the nfa and pentane-E 1:1 for the hfa. We have used Bio-Rad Laboratories "silicic acid for lipid chromatography" and Clarkson Chemical Co. "Unisil." The latter is faster running and needs little or no pressure, but it is more expensive. Unfortunately, there seems to be a slight loss of hfa on the column.

The methyl esters of nfa and hfa can be separated with a silica gel column using pentane containing 3% E and 20% E (31). Another solvent system is methylene chloride for nfa esters and methylene chloride containing 3% M for the hfa esters (102). B-M mixtures have also been used (19).

The esters can also be separated with a Florisil column using H and H-E 9:1 (59). Cholesterol comes off with the hfa. Florisil, a coprecipitated magnesia-silica gel (Floridin Co., Tallahassee, Fla.), has the advantage of being much less expensive than silica gel so it is particularly suited to large scale work.

Following the recommendation of Carroll (9), we now add water to the activated Florisil, since this allows use of lower volumes of eluting solvents. The Florisil is heated in 200 g batches in a Vycor beaker for 1 hr at 600C, cooled in a desiccator, and mixed gradually with W, 8 ml per 100 g. The hydrating mixture is stored 24 hr before using, with occasional stirring. It should be discarded after 3 months. This time restriction seems to be due to sudden deterioration in the moistened adsorbent (15). With this Florisil our solvents are H-B 9:1 and H-E 8:2; sphingosine is not eluted under these conditions. Carroll has performed the same separation with slightly drier Florisil, the nfa coming out with H-E 95:5 and the hfa, with H-E 75:25 (10).

The loading ratio which we use for both adsorbents is 70 mg adsorbent per mg of adsorbing material. The substances eluted with the first solvent, in which the sample is added and the column packed, are not included in this calculation. Elution volumes are roughly 25 ml/g of adsorbent. Each batch of adsorbent must be evaluated by TLC, of course.

While Downing et al. have found alumina to cause some hydrolysis of fa esters (24), others have claimed successful separations of nfa and hfa with such columns (12). The ω -hfa of sebum were separated from nfa (as the methyl esters) on alumina washed with hot 2% nitric acid (48). Hexane eluted the nfa and E the hfa. A similar separation of carnauba wax hfa has been accomplished with petroleum ether for the nfa esters and a succession of B-A, toluene-A, and C-HOAc for the hfa esters. The alumina, after acid washing, was activated at 500C for 5 hr and deactivated by storage over water (87).

When hfa occur together with α,ω -dicarboxy acids, they can be separated as the methyl esters on a silica gel column, using pentane-E 90-10 for the dicarboxy acids and pentane-E 70-30 for the hfa (95). A separation of three types of methyl esters has been described: nfa, epoxy fa, and hfa (80). A rather heavy loading ratio was used: about 18 mg silica gel (Davison) per mg of adsorbed ester. The nfa were eluted with H-E 97:3, the epoxy esters, with more of the same and also H-E 95:5, and the hfa, with plain E. A sharper separation was achieved by using segmented columns, in which the solvent enters successively narrower column segments. We follow the recommendation of Kabara (56) and use 1-piece glass columns in which the segments are of equal length but differ by a factor of about 1.4 in inside diameter.

It is rather surprising to learn that the above separations can be performed batchwise (80). The mixed esters were simply dissolved in petroleum ether-E 97:3 and mixed with silica gel, and the adsorbent was filtered and washed on the funnel. The filtrate contained almost pure nfa. Repetition of the extraction and washing of the silica gel with more polar mixtures gave rather pure epoxy fa. The hfa were not eluted but presumably could have been recovered. The filtration step, if performed with a cylindrical funnel, amounts to a column elution so perhaps the batch method is not any more convenient.

The separation of hfa from nfa has also been accomplished by first reducing the acids, as methyl esters, to diols and monohydric alcohols (24). Lithium aluminum hydride was used for the reduction, which is virtually quantitative, and the alcohols were separated with an alumina column. This separation is particularly interesting since it yielded three fractions from wool fat: monohydric alcohols (C-B 1:2),

α,ω -diols (C-A 2:1), and α,β -diols (more C-A 2:1). Additional specificity to the separation can be given by converting 1,2-diols to ketals, which are somewhat less polar. Downing et al. (24) converted the diols to hydrocarbons in good yields by reaction with iodine and red phosphorus, then with lithium aluminum hydride. This method, albeit lengthy, has the advantage that branched chain hfa are readily separated as the hydrocarbons and that GLC with the hydrocarbons is unusually efficient and especially useful for acids having very long chains.

It should be possible to shorten the above reductive approach by reducing the original hfa esters directly, without conversion to the methyl esters (47). However, the problem of removing nonsaponifiables might then be introduced. Cholesterol would probably elute with the monohydric alcohols formed from nfa. In the case of the h²fa, isolation in the form of 1,2-glycols would be useful if one wished to degrade the glycol with periodate for radioactivity work or quantitative determination.

Adsorption column chromatography has also been very effective for separating saturated from unsaturated hfa. This can be accomplished by converting the unsaturated hfa, as their methyl esters, to somewhat more polar compounds which are then readily separated with a Florisil or silica gel column. A suitable derivative is the methoxy mercuriacetate adduct, formed by warming the ester with mercuric acetate in M. After removal of the excess reagents by solvent partition, the mixture is added to the column. With Florisil (59), the saturated hfa esters are eluted with H-E 9:1 and the unsaturated esters with A-C-concd HCl 10:8:1. The strong acid regenerates the unsaturated ester and the A-C elutes it from the column. We have used this method also to separate saturated nfa and hfa, while discarding the unsaturated esters (41).

A similar separation with silica gel can no doubt be accomplished, but the published procedures appear to have been applied only to nfa esters (34,36,52). The mercury adducts can be eluted without decomposition and it is possible to achieve separation according to the number of double bonds, but the yields seem to fall off after the monoenes are eluted. The mercury adducts have also been separated with alumina columns, yielding monoenes and polyenes as separate fractions (68).

The problem of separating saturated from unsaturated hfa can also be handled with silver nitrate-silica gel (18). This adsorbent, by virtue of a weak, rapidly reversible reaction between silver ions and double bonds, allows separation according to the number of double bonds as well as the configuration of the bonds. No preliminary reaction is necessary, and elution is carried out in the usual way, with solvents of increasing polarity. The free OH of hfa esters does not interfere (15). We are currently using this adsorbent (25 g silver nitrate, 100 g silica gel) with H-B 8:2, which yields the saturated hfa methyl esters, and H-B 6:4, which yields the monoenoic hfa esters.

The main drawback to the silver columns is their unexpected tendency to separate esters according to their chain lengths (62). The separation is poor compared to GLC separations, but it does tend to produce overlapping fractions. The longer chain esters elute faster and we have been unable to separate h24:0 from 18:1 in attempts to separate the cerebroside total fa on a single silver column. Such

a separation has almost been achieved by Wagner et al. (116), but the procedure calls for close control of cut-off points, monitored by TLC or GLC.

An additional difficulty with the silver-containing adsorbent is the variability in quality one encounters from batch to batch. At present, the commercially available material gives poor separations and our attempts to prepare our own in large batches have also been unsuccessful. More research on how to prepare a good adsorbent of this type is definitely needed.

It should be noted that adsorption columns can often be used with unexpectedly large samples; that is, large amts of nfa can be removed from small amts of hfa with a small column. This is true only if the sample is added in the same solvent that is used to elute the nfa. Since the nfa under these conditions are adsorbed very poorly, the major load capacity of the adsorbent is usable for the more polar components of the mixture.

Separation of Hydroxy Fatty Acids by TLC

Separation of hfa from nfa by this means is fantastically simple and effective. This application of TLC for the methyl esters was first described by Vioque and Holman (115). The usual adsorbent, Silica Gel G, was used with H-E 85:15 and yielded excellent separations of dihydroxy fa, h⁶18:0, h¹⁴18:0, h²18:0, and nfa. However, it should be noted that TLC yields some separation according to chain length as well as degree of unsaturation (64), so the separations of natural mixtures may not be as good as with the usual demonstration test mixtures. As much as 50 mg of mixed esters can be separated on a single standard plate (80). The proportion of E in the H-E mixture can be raised to 40% to give improved resolution of polar esters.

The hfa ester spots, once located, can be eluted readily from the plates and used for further separations and determinations. We like a spray of bromthymol blue in dilute NaOH (53), which unlike iodine does not attack unsaturated esters or give low intensities with saturated esters. The hfa esters are readily eluted from the silica gel by packing the dry powder with E in a small column and eluting with more E. The indicator is not eluted.

The free nfa and hfa can also be separated by TLC by adding 1% acetic acid to the H-E mixture (71, 84). Other workers have raised the acetic acid concn to about 4% or used C-HOAc 96:4 (53). The acetic acid is necessary to prevent streaking of the free fa. Other solvents that have been used for the free hfa; H-EtOAc-HOAc 70:30:1 (93) and B-M-HOAc 45:8:4 (94). Recovery of the free hfa can be accomplished as above, eluting with E.

The separation of *unsaturated* fa esters has been accomplished by TLC versions of the two methods described in the previous section. Separation of the mercuric acetate adducts of nfa was achieved with silica gel containing only 1% plaster of Paris and two successive solvents: H-E 4:1 and propanol-acetic acid 100:1 (72). The spots were visualized with *s*-diphenylcarbazone, a mercury detector. The unsaturated esters were recovered by shaking the powder with M-conc. HCl 20:1 and extracting the solution with W-E. It is likely that better recoveries would be obtained if the powder were eluted after packing in a small column.

The silver nitrate-silica gel approach has found more use because of the elimination of the mercuric acetate condensation step (79,4). However, we have found as with the columns that chain length effects tend

to produce overlapping of spots (mainly trailing of unsaturated nfa into saturated hfa). Perhaps the use of two successive solvents would solve the problem. In the absence of nfa or of wide variation in chain lengths, both TLC approaches look quite suitable for hfa.

The prostaglandins, a family of unsaturated hfa containing a 5-membered ring and two or three OH groups, have been separated by TLC using solvents which may be of interest for other problems (38). For the free acids, B-dioxane-HOAc 20:20:1 was used with Silica Gel G and EtOAc-HOAc-M-2,2,4-trimethylpentane-W 110:30:35:10:100 was used with silver nitrate-silica gel plates. For the methyl esters, B-dioxane 5:4 was used with Silica Gel G and EtOAc-M-W 8:2:5 or 32:5:20 was used with the silver system.

In the current enthusiasm over TLC methods, perhaps we should not lose track of older methods. Separation of the mercuric acetate adducts of nfa and hfa has been accomplished on paper chromatograms (50) and separation of nfa from hfa has been achieved with silica gel impregnated paper (99). In the latter method, the position of the hydroxyl group influenced the R_F. Silicone impregnated paper, with W-acetonitrile 1:1, has yielded nice separations of nfa, epoxy fa, mono-hfa, and di-hfa methyl esters (84). The same combination of compounds, as the free acids, separated well with W-acetonitrile-acetic acid. Mineral oil impregnated paper has yielded similar separation, including separation of homologs and saturated and unsaturated fa (106).

Separation of Homologous Hydroxy Fatty Acids

Partial separations of the hfa, in the form of their O-acetyl derivatives, have been made with a reverse phase liquid column (31). In this system, as in similar systems, the separations are adequate for 2-carbon steps but not for adjacent or unsaturated homologs.

GLC is the method of choice, just as with the nfa. The first such separation was accomplished with the methyl esters after etherification of hfa with methyl iodide and silver oxide (59). It had previously been found that poor recoveries and poorly shaped peaks were obtained if the hydroxyl group was not masked. The ether formation, ordinarily considered a low-yield reaction, was pushed to completion by using the methyl iodide as solvent, by adding several portions of silver oxide, and by refluxing 3 hr. More recently, the ether has been made by reaction with methyl iodide and sodium hydride in dimethylsulfoxide (43,119). This reagent system appears to be much faster but its utility has not yet been conclusively documented. The ether esters can be separated by GLC in the same way as nfa esters. Because of the methoxyl group, they come off the chromatograph roughly 1.5 C-numbers after the corresponding nfa ester.

The OH group of hfa can also be masked by acetylation. We adapted the method of Hagemeyer and Hull (40,61), reaction with isopropenyl acetate (the acetate ester of acetone enol; Eastman Organic #6324). A few mg of the methyl hfa ester are deposited in a short conical test tube made from a 13 × 100 mm screw-cap culture tube, and 50 μl of isopropenyl acetate-sulfuric acid 500:1, freshly mixed, is added. The tube is capped and heated in a 60C bath for 30 min, then extracted with 0.3 ml W and 0.3 ml toluene-E 1:1. The extraction step hydrolyzes the excess reagent and removes the sulfuric acid, acetic acid, and acetone. The upper layer is exposed to a few grains of potassium carbonate to remove

residual water and acid and the esters are analyzed by GLC.

The acetylation method is faster than the methylation method but the GLC separations seem distinctly inferior and a higher column temp is necessary. However, if the hfa are to be isolated from the gas chromatograph and degraded chemically, the acetate derivative is preferable as it is much easier to hydrolyze.

We have shortened the acetylation method recently (15) to save the trouble of washing out the acid catalyst. The sample is heated as above with 0.4% toluenesulfonic acid in iso-propenyl acetate, then left with a crumb of potassium carbonate for 15 min. The supernatant solution is injected into the gas chromatograph directly, without further treatment. There is an unknown soluble byproduct formed from the reagent, so we have inserted a glass liner tube with a glass wool plug in the injection oven of the chromatograph. The tube is removed periodically and washed out.

In a recent paper on GLC of the hfa (90), O'Brien and Rouser have described the application of a Fritz-Schenk acetylation reagent (30), pyridine-acetic anhydride-toluenesulfonic acid. The reaction is accomplished in 3 min at 100C and the acetate is recovered by adding W and H. The ester solution is evaporated to dryness, then evaporated several times after additions of E (until the pyridine can no longer be smelled).

Apparently it is not necessary to modify the OH group for all gas chromatographic conditions. O'Brien and Rouser state (90) that saturated hfa shorter than 20 carbons need not be acetylated. Morris et al. state (83) that $h^{21}18:0$, $h^{12}18:0$, and methyl ricinoleate need not be acetylated. Preiss and Bloch (95) have isolated $h^{18}18:0$ and $h^{17}18:0$ as the methyl esters, using silicone XE60. Morrissette and Link (85) have obtained fine peaks with $h^{12}18:0$ and even dihydroxystearate, using the polyamide, Versamid 900. Gerstl et al. (33) analyzed brain h^2 fa up to $h^{26}:1$ without derivatizing. Miwa et al. (77) have compared the retention times of hfa (mono- and dihydroxy) with those of other fa on Apiezon L and Resoflex 446. Carroll (10) has used SE-30 for the h^2 fa methyl esters up to $h^{24}:0$ but found it necessary to use the methyl ethers when polar packings were used. The ethers ran only slightly faster than the free alcohols with SE-30.

Perhaps the use of all-glass columns, well siliconized, and lightly loaded packings obviates the need to mask the hydroxyl group. However, the advantage gained by decreasing the mol wt of the hfa may be lost, in terms of retention times, by the slowing effect of the free hydroxyl group.

Not all hfa can withstand the rigors of GLC. When the hydroxyl group is close to a double bond, dehydration takes place (82,83) and acetylation does not prevent this. In this study, the packing was 15% polyester; it may well be that the degradation problem could be overcome by reducing the packing concn somewhat. Also, the flash heater temp can be reduced somewhat if a temp program is used for the column.

We have made some tests with the trimethylsilyl ethers of hfa (64). They were readily formed with trimethylsilyl chloride and hexamethyldisilazane by the method of Sweeley et al. (110) and TLC showed the reaction went to completion. There was no particular improvement in retention times or peak sharp-

ness as compared with the acetate esters, nor was there a saving in processing time, since we evaporated the silylating reagents off with nitrogen and extracted the ether esters with H. For preparative scale GLC isolations it would seem wise to reduce the amts of the excess reagents injected into the chromatograph. Perhaps it is easier to regenerate the hfa after isolating the individual ether esters by GLC, for the ethers are quite unstable in acid. In a recently published abstract, Wood et al. (118) state that the trimethylsilyl ethers are eluted much faster than the acetate esters.

The trifluoroacetate derivatives of the hfa do not seem to have been investigated yet. It is likely that they would show shorter retention times. However, it appears to be difficult to obtain satisfactory trifluoroacetic anhydride.

Another approach to GLC isolations of the hfa, suitable for the 2- or 3-hydroxy acids, is via the reduction to diols and conversion to the acetonyl ketals (22). The hfa esters are reduced with lithium aluminum hydride, and exposure to acetone-sulfuric acid for 2 hr yields the ketals. This type of derivative is readily regenerated to diols for further degradation.

In the special case of the h^2 fa, one can decarboxylate the free acids with lead tetraacetate, a very specific reagent for glycols and h^2 fa, and analyze the resultant aldehydes by GLC (60). This method has the desirable feature of lowering the retention times considerably but the aldehydes cannot be stored very long prior to GLC. However, now that liquid nitrogen refrigerators (-196C) have become practical, the way is no doubt open to indefinite storage of the aldehydes.

Standards for GLC identification of hfa are rather scarce. A crude preparation of $h^{21}18:0$ acid is available from K & K Laboratories (Plainview, N. Y.), which also sells "1-hydroxypentadecanoic acid-15" and 9 (10)-hydroxystearic, $h^{12}18:0$, and $h^{26}:0$ acids. Applied Science Laboratories (State College, Pa.) sells more highly purified hfa: $h^{21}14:0$ and $h^{12}18:0$ acids and their methyl esters. Lachat Chemicals (Chicago, Ill.) offers rather pure $h^{26}:0$, $h^{12}18:0$, and ricinoleic acids. The 2-hydroxy fa are simple to make, by successive reaction with thionyl chloride, bromine, and aqueous KOH (86,103), and one can make an entire homologous series by starting with a mixture of nfa. Of course one can always use a natural preparation of hfa if the major peaks have already been identified by someone else.

Methods of Structure Determination

Locating the Hydroxyl Group

If the OH is in the 2-position, it should be possible to precipitate the copper chelate, as described earlier. The precipitate is a very pale blue, whereas nfa or fa in which the OH is further from the COOH, yield a deep blue copper salt under ordinary conditions of precipitation.

If there is no confusion arising from homologs, one can show by the R_F on TLC (113) or silica gel paper (99) chromatography that a 2-OH or 3-OH fa is present. Since the differences are not very great, a closely similar standard is necessary. If the hfa is reduced to a 1,2-glycol, it can be identified as one by the periodate reaction, which yields formaldehyde, a readily identified product. If the resultant glycol forms an acetonyl ketal, the hfa must have been a h^2 fa or h^3 fa.

If, in the course of the manipulations, the hfa has formed a lactone, one may assume the OH group is

and dicarboxy acids, as is usually done. By analyzing the two groups of acids together, one can more readily quantitate their peak areas and determine whether a given peak is due to overoxidation or to the presence of a fa isomer with a double bond in another position. (3) Unlike the reductive cleavage methods, where aldehydes and aldehyde esters are formed from the ozonides, the products are stable and there is less difficulty in obtaining standards for GLC identification. (4) No complex apparatus or unusual catalyst is needed. As with all oxidative degradations, some byproducts are formed and one must examine the resultant esters with two types of GLC columns in order to quantitate the various peaks. This question is particularly important when the original compound is a mixture of isomers. We found 3 to 4 isomers in each brain monoenoic hfa.

The need for standard methoxy dicarboxy esters for GLC comparison can be a problem. We made the 2-methoxy compounds by mixing several dicarboxy acids, brominating with only one mole of bromine, hydrolyzing to the hfa, and methylating. At each stage the products were purified by copper precipitation or Florisil columns and examined by TLC.

The effective periodate-permanganate method of splitting double bonds has also been applied to hfa (37). A yeast monoenoic hfa was cleaved in this way, without protecting the OH group, and yielded nonanedioic acid and a h9:0. The acids were separated by preparative GLC in the form of the butyl esters, since the methyl esters were poorly resolved. Finding nonanedioic acid showed the double bond was in the 9-10 position. Identification of the h9:0 as h⁸9:0 was made by oxidation to the keto acid and comparison with the known keto acid.

Ozonolysis can also be applied to hfa without protection of the OH group. Reductive cleavage of the ozonide yields a hydroxy aldehyde which might be useful for further characterization. Dimorphecolic acid was treated this way, after reduction of the ester to an alcohol (lithium aluminum hydride). Reductive ozonolysis yielded hexanaldehyde and a dihydroxy-aldehyde which reacted with dinitrophenylhydrazine to form an osazone (108). The osazone formation showed that the OH of the original acid was adjacent to one of the double bonds and that it was between this double bond and the carboxyl group.

While the configuration of a double bond can generally be identified as *trans* by the absorption peak at about 10.3 μ , there are cases in which this peak is split or assignable to another function. This problem arose in the identification of the hfa of royal jelly, 10-hydroxydec-2-enoic acid (2). This acid showed a peak at 10.1, but its hydrogenation product showed a similar peak at 10.2 μ . The double bond was established as *trans* by means of nuclear magnetic resonance analysis (3).

Cis double bonds show a small peak at 6.1 μ and either type shows a small, sharp peak at 3.3 μ , whose detection requires an instrument with good resolution in this region.

Quantitation of the Hydroxy Fatty Acids

Gravimetric

This approach can obviously be applied to any sample of pure hfa isolated by one of the methods described in the first section of this article. As other methods were developed, this approach lost favor with the analytical chemist. However, certain recent developments make it once more very attractive. First,

the efficiency of separation that can be achieved with modern adsorbents makes it relatively easy, in many cases, to isolate very pure hfa. The blanks from these adsorbents are generally very small. Second, the invention of the Cahn Electrobalance (Cahn Instrument Co., Paramount, Calif.) has made extension of gravimetry to the milligram level very easy.

The usefulness of the Electrobalance has recently been augmented for the lipid chemist by the introduction of small Teflon weighing cups (49) which hold 2 ml. Samples of 1 mg or less can be weighed very accurately and quickly. One can evaporate a chromatographic fraction to dryness in a small flask, then transfer the hfa with three 1-ml portions of benzene, letting the solvent evaporate in the cup between rinses (16). Use of a more volatile solvent leads to unpleasant creeping.

Since one can readily purify several mg of hfa on a single TLC plate and elute the material from the powder, combination of the two techniques may be the answer to many analytical problems. Unlike other general methods of determining fa (titration, charring, liquid/liquid partitioning with copper or dyes, etc.), the gravimetric method gives samples which can be readily processed further.

Gravimetric determination of decigram quantities or more can lead to high values because of the difficulty of removing solvent. While classical organic chemistry offers reasonable methods of drying, we would like to recommend lyophilization for solid lipids. We routinely evaporate the samples to a syrup by rotary vacuum distillation, then displace the remainder of the solvent with benzene by addition of benzene and further evaporation. A little more benzene is added and the sample is frozen and lyophilized in the usual way. This method gives a fluffy product which is free of solvent, and is reasonably fast and safe from the dangers of splashing resulting from attempts at complete drying by vacuum evaporation.

Colorimetric

In the case of the h²fa, one can precipitate the copper chelate and measure the amt of copper in the chelate. This can be done simply by dissolving the chelate in a chromogenic chelator, diethyldithiocarbamic acid (disodium salt), which forms an intense color with copper ions (61).

There are a number of methods for colorimetric determination of hydroxy compounds which do not seem to have been tried with hfa but which should work. A sensitive and reasonably rapid method involves acetylation, hydrolysis of the excess acetic anhydride, treatment with hydroxylamine to form the acethydroxamate, and color formation with ferric perchlorate (39,78). Only about 3 μ moles of hydroxy compound are needed and all the steps involve simple additions of liquid to a container.

Another method involves the reaction of the hydroxy compound with 3,5-dinitrobenzoyl chloride in pyridine, partition between H and aqueous HCl to remove the reagents, and color formation by addition of dimethylformamide and propylenediamine (54). In another version, for hydroxysteroids, the color is developed in acetone-aqueous KOH (13). The reaction is very sensitive. A similar approach is the formation of the *p*-nitrobenzoyl ester, removal of the excess reagent, and determination of the aromatic ester by its ultraviolet absorption (103a).

If the hfa is separated as the free acid from its contaminants, one has available a variety of methods for determining the carboxyl group. The transfer of cupric ions into an organic phase is the basis of a sensitive, elegant method for nfa (25), but it remains to be seen whether hfa do not give trouble because of solubility differences. If the hfa is separated as the methyl ester from other esters, one can use the hydroxylamine-ferric ion method. In our experience with h²fa, this reaction requires extra time as compared with the nfa but can be made reliable by using a controlled temp (35).

It is possible to oxidize secondary alcohols to ketones with chromic acid, then to determine the ketones with dinitrophenylhydrazine, using KOH to develop the color (14). The oxidant must be removed with hypophosphorous acid before forming the hydrazone. It would be nicer if one could determine the amount of reduced dichromate colorimetrically, but I suspect that side reactions would make this impractical.

Gas-Liquid Chromatography

The hfa seem to behave much like the nfa in GLC with thermal conductivity detectors. In the case of argon ionization detectors, the hfa acetates give only one-half the response of nfa (90). Unfortunately, mixtures of hfa for testing an instrument's ability to quantitate are not yet available.

Other aspects of hfa GLC are discussed in an earlier section, under Isolation Methods.

IR Absorption

Quantitative analysis by IR spectrophotometry is not yet a popular method, but it should be seriously considered for the hfa in view of the distinct and fairly strong OH peak at around 2.9 μ . Generally the OH of carboxyl groups interferes so it is better to work with the hfa esters. The OH groups of water, alcohols, and peroxides also interfere. Almost any solvent can be used to dissolve the hfa ester. The solvent is placed in the reference cell and the solution in the other cell, and the spectrum is recorded at a slow scanning speed in the region of the OH peak. Details of calculation, which are a little different because the sample has some basal absorption at every wavelength, may be found in books on IR work.

There are a number of good reasons why this approach is not used by as many people as own IR spectrophotometers. At best, high precision cannot be obtained with present day instruments. The method is hardly as sensitive as most methods, in terms of sample size. Hydroxy compounds tend to show effects of association, depending on the solvent; that is, the specific absorption depends on the concn. Pyridine has been used to counteract this effect (57). The sample temp (which rises with time) affects the sample's absorptivity. Thermostatic cells are now available, but their effectiveness is yet to be determined.

The value of the approach lies in its simplicity: preparation and purification need only be minimal. Moreover, a continuous flow cell could be used to follow changes in a stream. IR determinations of hfa have been described by Thomas (112) and Ahlers and McTaggart (1) and are discussed in O'Connor's reviews (91,92).

IR absorption has been used to determine hfa in which a double bond is present in an allylic position, for the OH absorption peak disappears on treatment with acid (81).

Determination by Titration

The standard method for hfa is acetylation followed by extraction of the acetate and determination of its saponification equivalent. The difference between this value and that of an unacetylated aliquot gives the number of equivalents of hydroxyl group. In general, saponification equivalents suffer in precision because one must titrate the excess alkali before and after saponification. A more precise method involves passing the saponified solution through an acid cation exchanger (Dowex 50-H⁺) and titrating the effluent directly with alkali. Thus one aliquot gives the total number of fatty acid carboxyl groups, while the aliquot from the acetylated sample gives the total number of fatty acid *and* acetate carboxyl groups. The difference between the two values gives the number of acetate groups. Only two titrations, instead of three, are involved in this method.

When large amts of nfa are also present, even this modification is imprecise and one can then make a direct acetyl group determination. Generally this is done by saponifying or hydrolyzing the acetate ester, then steam-distilling the acetic acid, and titrating the distillate. Texts on quantitative organic microanalysis describe such procedures. A high concn of magnesium sulfate in the hydrolysate speeds the distillation of the acetic acid (29).

The fastest version of the acetylation methods involves acid-catalyzed acylation and direct titration of the excess acetylating agent. A recent description of such a method, intended primarily for high mol wt alcohols (55), calls for a mixture of acetic anhydride in perchloric acid, ethyl acetate, and hexane. After 5-10 min, the excess reagent is hydrolyzed with pyridine-W and the acetic acid is titrated visually or potentiometrically. This method does not require removal of nfa, but it does require adjustment of reagent volumes in proportion to sample size if good precision is to be maintained. A microversion of the same basic method is given by Schenk and Santiago (101).

We have used titration with (ethylenedinitrilo) tetraacetic acid to determine the copper content of precipitated h²fa (61).

Radiometric Determination

By forming a specific radioactive derivative of the hfa, one can measure the amt present simply by counting. All that is needed is a knowledge of the specific activity of the derivatizing reagent and a feeling of assurance that the reaction is complete and that the reagent can be removed completely. Tritium- or C¹⁴-labeled acetic anhydride is used in this way to determine hydroxysteroids at very low levels (67). The acetylation is generally done in pyridine and the reagents are removed by solvent partitioning and vacuum evaporation. If a liquid scintillation counter is available, counting can be done very simply even when large amts of nfa and other nonhydroxy compounds are present. One should be able to achieve a precision of 1 or 2%. It is not necessary to count the reagent in order to determine its specific activity: simply acetylate a known weight of a pure standard, such as cholesterol, and measure the activity of the product. The calculation (for a monohydric alcohol) is:

$$\frac{\text{micromoles unknown}}{\text{cpm of unknown}} = \frac{\text{micromoles standard}}{\text{cpm of standard}}$$

Incidentally, if the hfa is first isolated chromato-

graphically and weighed, its mol wt (or equivalent weight) can be determined by this method.

A potentially valuable variation on the acetylation method of radiometric determination has been described by Karmen et al. (58). A known weight of a standard hydroxy compound is added to the impure unknown (hydroxysteroid) and the mixture is acetylated with labeled anhydride. A portion of the esters is then subjected to GLC and the peaks (including the peak from the standard) are collected and counted. Comparison of the total activities and weight of standard yields the original amt of the unknown. The method allows determination of several hydroxy compounds in a single mixture, does not require uniform response by the GLC detector, and does not require detectable weights, since nonradioactive acetylated carrier can be added after the acetylation step.

Another radiometric approach is the formation of tritium-labeled methyl esters (from dry HCl in labeled M), followed by TLC, elution of the hfa spot, and counting. This is a very sensitive method; visualization can be aided with carrier. Unlike the acetylation methods, this one allows determination also of the nfa and any other fatty acids that can be separated on the plate.

Other Methods

The researcher dissatisfied with the methods described above can consider other methods which have been used for compounds containing hydroxy groups. Active hydrogen determinations should be useful with hfa esters as long as peroxidized esters are not present and the sample is bone dry. We have tried measuring the amt of carbon dioxide produced by oxidizing h²fa, but always found high values, apparently due to attack on the solvents (42). Curiously, the solvent blanks were low; I believe the inorganic byproducts from the oxidant catalyzed oxidation of the solvent. Quantitative TLC by the charring method, described by Privett in this Symposium, should be equally applicable to the hfa.

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The Metabolism of Polyenoic Fatty Acids

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Abstract

Feeding experiments with C¹⁴-labeled and unlabeled unsaturated fatty acids have been used to study the possible routes of formation of the C₂₀- and C₂₂-polyenoic fatty acids of rat liver phosphatides. The acids of the palmitoleate, oleate, linoleate, and linolenate types (considered on the basis of the position of the double bond closest to the methyl end) are apparently formed from the C₁₆ and C₁₈ unsaturated acids of the corresponding types. The results rule out possible transformations of the C₂₀- and C₂₂-polyenoic acids from one type to another, and demonstrate the exclusive introduction of new double bonds toward the carboxyl group. Isomers of linoleate or linolenate in which the double bonds were shifted by one carbon atom toward the carboxyl or methyl groups were incorporated into the phosphatides only to a negligible extent in the form of polyenoic acids.

FIG. 1

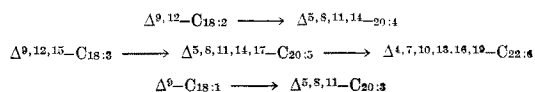


FIG. 2

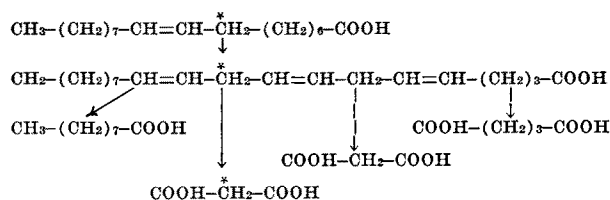
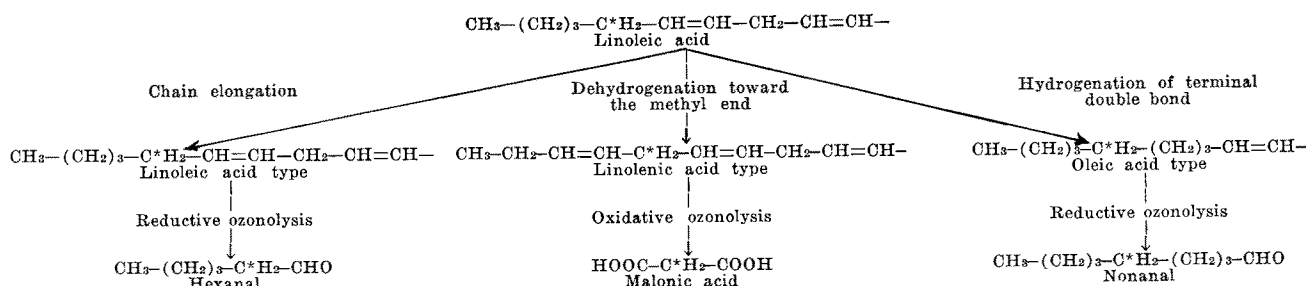


FIG. 3



MOST OF THE C₂₀- and C₂₂-polyenoic fatty acids occurring in the phosphatides of the higher animals have their first double bond (counted from the terminal methyl group) in the 6 or 3 position. Linoleic acid, the C₁₈-diene, has its first double bond in the 6 position and linolenic acid, the C₁₈-triene, has its first double bond in the 3 position. Thus the larger polyenoic acids may be considered as being either linoleic or linolenic acid types (Fig. 1). The latter acids have been shown to be precursors of the longer acids, undergoing chain-elongation and introduction of new double bonds (1). The new double bonds are directed toward the carboxyl group and maintain the divinyl-methane rhythm. The enzyme system carrying out these reactions is found in the liver microsomal fraction, as shown by Stoffel (2).

Besides the long polyenoic acids of the above two types, we have found in phosphatides a smaller amt of 20:3^{5,8,11}, with a structure of the oleic acid type, the first double bond being in the 9 position (3,4). It has been known for some time that a C₂₀-trienoic acid accumulates in liver lipids of rats on a fat-free diet. Mead and colleagues (5) have identified this acid as being 20:3^{5,8,11} and have given evidence for its synthesis from acetate (6). The reasonable assumption has been made that stearic and oleic acids are intermediates in the biosynthesis. We have given direct evidence for the transformation of oleic acid into this triene by feeding 8-C¹⁴-oleate to fat-deficient rats (7). The labeled fatty acid was made by total synthesis. Both the C₂₀-triene and arachidonic acid were isolated from liver. The trienoic acid had a specific activity of 8730 dpm/mg and 96% of its radioactivity was located in the malonic acid obtained by oxidative ozonolysis. The nature of the reactions is illustrated in greater detail in Figure 2.

On the other hand, the C₂₀-tetraenoic acid of the linoleic acid type, arachidonic acid, had a specific activity of only 115 dpm/mg. Thus only the 20:3^{5,8,11}