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Nonphosphatide aldehydogenic lipids in milk fat, beef tallow, and ox heart

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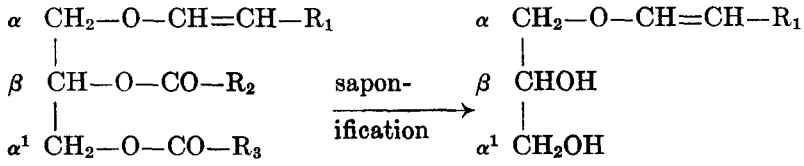
SUMMARY

Phospholipid-free milk fat, beef tallow, and ox heart fat contain approximately 50 (calculated as tetradecanal), 65, and 1000 mg per kg (calculated as hexadecanal), respectively, of aldehydes. The aldehydes are bound as enol-ethers, and are located mostly in the α-position of the glycerol molecule. A sample of milk fat was found to contain 45 mg per kg glycerol ether (calculated as chimyl alcohol).

In recent years the chemical structure of the plasmalogens has been the subject of a number of investigations. Klenk and Debuch (1) proposed, as one of several alternatives, the structure in which an aldehyde is linked by an enol-ether bond to the glycerol moiety. Evidence for this structure is rapidly accumulating (2 to 5), but the position of the aldehyde on the α- or β-carbon of glycerol is still a matter of controversy (6, 7, 8). Karnovsky and co-workers (9) reported the occurrence of acetone-soluble, aldehyde-containing lipids in the starfish (*Asterias Forbesi*). This led them to consider the possible existence of non-phosphorus-containing lipids that split off aldehydes in acid.

This paper presents experimental evidence for the occurrence of "nonphosphatide aldehyde-containing lipids" in ox heart, beef tallow, and bovine milk fat. Our starting point was the same as Karnovsky's, namely, the observation that lipid material not containing phosphorus could split off aldehydes. As a working hypothesis, we drew up a formula analogous to plasmalogen:



This material, when saponified, should give two fatty acids and an α-glycerol enol-ether. Experimental evidence supporting this structure is presented. In the course of the investigation, saturated α-glycerol ethers of the batyl alcohol type were detected in cream.

METHODS AND RESULTS

Starting Materials. Milk fat was obtained by churning 35% fresh cream in a Hobart-mixer at 15°, after which the butter obtained was melted at 50°. The clear fat layer was dried with anhydrous $MgSO_4$ and filtered. The resulting fat did not contain detectable amounts of phosphorus.

The absence of phosphorus was checked by the method of Zilversmit and Davis (10). Two g of lipid were incinerated in the presence of magnesium carbonate, and the ash gave a negative phosphorus reaction. By the above-mentioned method, 2 μg of phosphorus is easily detected, which implies that the lipids contain less than 0.0001% phosphorus.

Beef tallow was rendered from the minced tissue by careful melting at 120° under nitrogen. The dried and filtered tallow was subjected to countercurrent fractionation with isooctane-methanol 1/1(v/v). The triglyceride fraction contained no phospholipids.

Phosphorus-free ox heart lipids were obtained in

the following way. One kg ox heart was extracted by the method of Folch *et al.* (11); 32 g lipids were obtained and dialyzed through a rubber membrane by the method of Van Beers *et al.* (12). The phospholipids remained in the residue, while triglycerides, cholesterol, and cholesterol esters quickly passed into the dialyzate. Traces of phosphorus-containing compounds, which might have leaked through the membrane, were eliminated by passing the residue of the dialyzate, dissolved in ethyl ether, through a 20 × 450 mm silica gel column (13) with silica gel prepared according to Gordon *et al.* (14). The nonphosphatide lipids were eluted with 500 ml ethyl ether. In this way an 8 g lipid preparation, free from phosphorus, was obtained from 1 kg ox heart.

Determination of Bound Aldehydes with Three Methods. To eliminate free aldehyde, the lipids were heated at 140° for 6 hours and a pressure of 10^{-3} micron by the method of De Bruyn (15). Subsequently, the bound aldehydes were determined by conversion with methanol-hydrochloric acid to methyl esters, glycerol, and dimethyl acetals (16). After saponification and extraction, the dimethyl acetals in the unsaponifiable fraction were decomposed with 15 ml acetic acid for 16 hours at 50°. The free aldehydes were converted into their dinitrophenylhydrazones on the reaction column of Haverkamp Begemann and de Jong (17) and subsequently analyzed on the nitromethane SiO_2 partition column described by Kramer and van Duin (18). The dinitrophenylhydrazones of aldehydes with less than 12 carbon atoms, originating from contaminants in the solvents or from oxidative decomposition products, were discarded; those of aldehydes with more than 11 carbon atoms were determined spectrophotometrically in chloroform (max. 358 $m\mu$, and $\epsilon = 22500$ were used for the calculation). Milk fat contained 50 aldehydes per kg (calculated as tetradecanal);[1] beef tallow, 65 mg per kg, and ox heart phosphorus-free lipids, 1000 mg per kg (calculated as hexadecanal).

In the second procedure, milk fat, from which free aldehydes had been removed by high vacuum heating, was stirred with 2 parts glacial acetic acid and 2 parts 2 N hydrochloric acid for 2 hours at 50°. The fat was washed acid-free with water, dried over $MgSO_4$, and again subjected to high vacuum heating. The higher aldehydes in the distillate, determined as before, amounted to 45 mg per kg.

By the third method, 390 g milk fat, containing 42 mg per kg bound aldehydes according to the previous method, was saponified with 135 g KOH in 1.35 liters ethanol by refluxing for 1 hour. After diluting with 6.5 liters water, the unsaponifiable fraction was successively extracted with 2, 1, and 1 liter portions of ether. The unsaponifiable fraction containing 8.2 mg aldehyde, i.e., 50% of that originally present, was then subjected to a 100-tube countercurrent fractionation with isooctane-methanol 1/1(v/v). The fraction corresponding to monopalmitin in a model experiment (partition coefficient 0.1) was isolated. Free aldehydes, if any, were converted to acids by oxidation with silver oxide in 90% dioxane, according to the method of Mitchell and Smith (19), and the acids extracted with alkali. The unconverted neutral portion was taken up in optically pure petroleum ether (b.p. 40°-60°), after which an aliquot was transferred to an 11 × 270 mm column of Celite® (Johns-Manville 545) loaded with 50% 2 N hydrochloric acid. Subsequently, 15 ml optically pure petroleum ether (20) was passed through, followed by 40 ml water. The aldehydes of the total unsaponifiable fraction in the petroleum ether eluate, determined as their dinitrophenylhydrazones, amounted to 7.2 mg. In the aqueous eluate, 3.7 mg glycerol was found by periodic acid oxidation, according to Hanahan and Olley (21). If 1 mole aldehyde were bound to 1 mole glycerol, 3.1 mg could be expected. That only glycerol was determined was confirmed by the paper chromatographic method of Hough (22).

Thirty-four g phosphorus-free lipids from ox heart, containing about 1000 mg aldehyde per kg, as determined by Leupold's method, was saponified with 12 g KOH and 120 ml ethanol. After diluting with 570 ml water, the unsaponifiable fraction was isolated by extracting the soap solution 7 times with ether (once with 560 ml; 6 times with 300 ml). The unsaponifiable fraction, containing 29 mg aldehyde (calculated as hexadecanal), was not subjected to countercurrent distribution but was immediately treated with silver oxide in order to remove free aldehydes. The unconverted fraction was isolated and subsequently taken up in optically pure petroleum ether. Of this solution, a 0.4 portion was transferred to a Celite®-hydrochloric acid reaction column (see above). In an aliquot of the petroleum ether eluate, the aldehydes were converted into their dinitrophenylhydrazones on a Celite®-dinitrophenylhydrazine column. Because the unsaponifiable fraction had not been fractionated by countercurrent distribution, the dinitrophenylhydrazone mixture still contained relatively large amounts of sterols which were removed by passing through a 15 × 170

[1] The composition of the aldehyde mixture will be published separately.

mm aluminum oxide column.[2] The hydrazones were eluted with 150 ml petroleum ether-ether 90/10(v/v). Only then were the dinitrophenylhydrazones analyzed on the nitromethane-silica gel column, yielding 9.7 mg aldehyde, which is 24 mg per total unsaponifiable fraction. A glycerol determination yielded 8.8 mg per total unsaponifiable fraction. If 1 mole aldehyde were bound to 1 mole glycerol, 9.3 mg glycerol could be expected.

Nature of the Bond Between Aldehyde and Glycerol. Apparently the unsaponifiable fraction of milk and ox heart fat contain compounds with 1 mole aldehyde bound to 1 mole glycerol. In view of the structure of plasmalogens, it is logical to suppose that the compound is an enol-ether. This can be proved by hydrogenation yielding glycerol ethers which, on treatment with acid, are no longer able to split off aldehyde (2, 4, 8).

Half the "monoglyceride peak" from the unsaponifiable fraction of 390 g milk fat, and from which the free aldehydes had been removed by oxidation, was dissolved in 3 ml methanol, and, after adding 40 mg of Adams' PtO_2 catalyst, hydrogenated at room temperature in 20 minutes. After taking up the product in optically pure petroleum ether, the solution was transferred to the Celite®-dinitrophenylhydrazine column, and 0.122 mg aldehyde was found; that is, 3.4% of the aldehyde originally present.

Of the unsaponifiable fraction from 34 g ox heart phosphorus- and aldehyde-free lipids, 4% were hydrogenated in the same way as above. From the hydrogenated product, 0.030 mg aldehyde (3.2% of the initial amount) was liberated on the Celite®-dinitrophenylhydrazine column.

Position of the Aldehyde Group in the Aldehyde-Glycerol Compound. To establish whether the aldehyde is at the α- or β-position, the aldehyde-glycerol compound was hydrogenated, and the glycerol ether so formed was treated with periodic acid according to Marinetti *et al.* (4). However, to draw a conclusion from such an experiment, it must first be ascertained whether glycerol ethers are present in milk fat before hydrogenation. To this end, a portion of the monoglyceride peak from the unsaponifiable fraction of milk fat, containing 4.46 mg bound aldehyde, was dissolved in petroleum ether and passed through a Celite®-hydrochloric acid column. The split-off glycerol remained in the column while the liberated aldehydes, together with any glycerol ethers present and other unidentified substances, were eluted with 40 ml petroleum ether, followed by 20 ml ethyl ether. The eluate was washed with water and sodium carbonate solution, evaporated, and the residue chromatographed on a 11 × 110 mm SiO_2 column (14) loaded with 5% water.

It had previously been established that 200 ml 1% ethyl ether in petroleum ether elutes aldehydes; 100 ml 25% ethyl ether in petroleum ether elutes cholesterol; and finally, 50 ml 100% ethyl ether elutes glycerol ethers.

The fraction emerging from the column in the position of glycerol ether, and no longer containing glycerol enol-ethers, was dissolved in glacial acetic acid. One-fourth of this solution was oxidized with periodate according to the method of Marinetti *et al.* (4). The amount of periodate consumed was determined spectrophotometrically and found to be 3 μmoles. Pure glycerol ethers consume 0.93 mole periodate per mole ether. In a portion of unsaponifiable fraction containing 4.46 mg bound aldehydes, 13 μmoles glycerol ether[3] was found.

Another equally large portion of the "monoglyceride fraction" from the unsaponifiable fraction of milk fat containing 4.46 mg bound aldehyde (calculated as tetradecanal) was hydrogenated, after which 3.4% aldehyde precursors still remained. The reduction product was passed through a Celite®-hydrochloric acid reaction column, and the eluate chromatographed over the SiO_2-5% H_2O column in the same way as the nonhydrogenated portion. One-fourth part of the glycerol ether fraction was again treated with periodate; 6.5 moles was consumed, corresponding to 28 μmoles glycerol ether. Thus 15 μmoles α-glycerol ether was formed during hydrogenation. The precursor of 4.46 mg aldehyde, which was 96.6% hydrogenated, should yield 20 μmoles. The difference could be due to β-glycerol ether.

DISCUSSION

In the unsaponifiable fraction of milk fat, only 50% of the amount of aldehydes determined in the whole fat by the Leupold method was found. It is possible that the missing aldehydes are present in the unsaponifiable fraction as normal acetal. Only 1.2% of the glycerol acetal of tetradecanal is split on the Celite®-dinitrophenylhydrazine reaction column. It is more probable, however, that the aldehyde-glycerol compounds were not completely recovered from the

[2] Alumina ICI, dried at 160°, to which 8% water had been added.

[3] The composition of the glycerol ether mixture will be published separately.

saponified lipid. According to Karnovsky *et al.* (23), glycerol ethers are difficult to extract from the soap solution even with ethyl ether. In this connection it should be mentioned that the recovery of the unsaponifiable fraction from ox heart lipid was more successful. This fraction was obtained by extracting the soap solution 7 times with ethyl ether, whereas the unsaponifiable fraction from milk fat was isolated by extracting only 3 times with smaller amounts of ethyl ether.

As has already been stated, the clear milk fat was heated to remove free aldehyde. In different portions of milk fat 0.1 to 0.8 mg free aldehyde per kg fat was found in the distillate, while about 50 mg per kg bound aldehyde was present. Usually a very small content of free aldehydes (dodecanal and higher) is also present in butter. For some samples of fresh butter, however, this content was higher than normal: in a Dutch butter, 20 mg per kg was found; in a German butter, prepared from sweet cream, 11 mg per kg. Whether these free aldehydes are formed from the plasmalogens or from the phosphorus-free aldehyde containing lipids is not yet known.

According to Van Duin (24), higher aldehydes in fresh butter are formed from the butter plasmalogens under the influence of the low pH of the butter serum. Aldehydes can probably also be split off by the action of enzymes or metal complexes (25).

Whether the glycerol ethers, the presence of which was demonstrated in the unsaponifiable fraction of butter fat, were present as such in butter, or were initially bound as glycerol ether fatty-acid ester, was not established.

This latter class of compounds was found by André and Bloch (26) in shark liver oil, and by Karnovsky and Brumm (27) in the starfish. It is unlikely that the glycerol ethers originate from a phosphatide, as the phosphoric acid component is not split off by alkaline saponification and the compound, from which only the fatty acid component is removed, does not, in consequence, enter the unsaponifiable fraction. In order to ascertain whether the glycerol ethers of milk fat occur as such or are bound, it will be necessary to subject the milk fat to countercurrent distribution and investigate the "monoglyceride" fraction.

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REFERENCES

1. Klenk, E., and H. Debuch. *Z. physiol. Chem., Hoppe-Seyler's* **296**: 179, 1954; **299**: 66, 1955.
2. Rapport, M. M., B. Lerner, N. Alonzo, and R. E. Franzl. *J. Biol. Chem.* **225**: 859, 1957.
3. Rapport, M. M., and R. E. Franzl. *J. Neurochem.* **1**: 303, 1956–57.
4. Marinetti, G. V., J. Erbland and E. Stotz. *J. Am. Chem.* Soc. **80**: 1624, 1958.
5. Blietz, R. J. *Z. physiol. Chem., Hoppe-Seyler's* **310**: 120, 1958.
6. Gray, G. M. *Biochem. J.* **67**: 26 P, 1957; **70**: 425, 1958.
7. Rapport, M. M., and R. E. Franzl. *J. Biol. Chem.* **225**: 851, 1957.
8. Marinetti, G. V., J. Erbland and E. Stotz. *J. Am. Chem. Soc.* **81**: 861, 1959.
9. Karnovsky, M. L., S. S. Jeffrey, M. S. Thompson, and H. W. Deane. *J. Biophys. Biochem. Cytol.* **1**: 173, 1955.
10. Zilversmit, D. B., and A. K. Davis. *J. Lab. Clin. Med.* **35**: 155, 1950.
11. Folch, J., M. Lees and G. H. Sloane Stanley. *J. Biol. Chem.* **226**: 497, 1957.
12. Van Beers, G. J., H. de Iongh and J. Boldingh. In *Essential Fatty Acids,* edited by H. M. Sinclair, London, 1958, p. 43.
13. Laroche, G. Thesis, University of Washington, 1957.
14. Gordon, A. H., A. J. P. Martin and R. L. M. Synge. *Biochem. J.* **37**: 80, 1943.
15. De Bruyn, J. *J. Am. Oil Chemists' Soc.,* in press.
16. Leupold, F. *Z. physiol. Chem., Hoppe-Seyler's* **285**: 182, 1950.
17. Haverkamp Begemann, P., and K. de Jong. *Rec. trav. chim.* **78**: 275, 1959.
18. Kramer, P. J. G., and H. van Duin. *Rec. trav. chim.* **73**: 63, 1954.
19. Mitchell, J., and D. M. Smith. *Anal. Chem.* **22**: 746, 1950.
20. Van der Ven, B., and A. P. de Jonge. *Rec. trav. chim.* **76**: 169, 1957.
21. Hanahan, D. J., and J. N. Olley. *J. Biol. Chem.* **231**: 815, 1958.
22. Hough, L. *Nature* **165**: 400, 1950.
23. Karnovsky, M. L., W. S. Rapson and M. Black. *J. Soc. Chem. Ind. (London)* **66**: 95, 1947.
24. Van Duin, H. *Neth. Milk Dairy J.* **12**: 90, 1958.
25. Thiele, O. W. *Z. physiol. Chem., Hoppe-Seyler's* **300**: 131, 1955; *IV. Kongress für Biochemie* (Sept. 1-6, 1958), pp. 18-38.
26. André, E., and A. Bloch. *Bull. soc. chim. France* **V2**: 789, 1935.
27. Karnovsky, M. L., and A. F. Brumm. *J. Biol. Chem.* **216**: 689, 1955.