In actual practice, triglyceride compositions are usually determined in mole percent values. A plot of $f_m$ vs. carbon number is made for the saturated monoacid triglycerides and the appropriate curve is drawn (Fig. 12). The $f_m$ values for mixed-acid triglycerides (i.e. $C_{40}$, $C_{50}$, $C_{52}$, etc.) are read from the graph. All saturated triglycerides of the same carbon number are assumed to have the same $f_m$ value. Where the $f_m$ values for saturated and unsaturated triglycerides of the same carbon number are different, an average $f_m$ value is assigned to each peak based on its estimated fatty acid composition.

The above considerations emphasize that *accurate calibration is essential for quantitative GLC of triglycerides.* Since calibration factors vary substantially with the operating conditions and the chromatograph used, the $f_m$ and $f_w$ values given in Table II do not necessarily apply to other laboratories. Even with the same instrument, column, and operating conditions, we have found that calibration factors vary slightly over a period of weeks.

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# Determination of Hydroxy-Acid Triglycerides and Lactones in Butter[1]

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## Abstract

The free and esterified ("bound") hydroxy acids from butterfat were isolated by column- and thin-layer chromatography on silica. The 4- and 5-hydroxy acids forming the γ- and δ-lactones were separated from the other hydroxy acids by TLC on silica. The concentrations of a number of free and bound lactones in butterfat were determined by means of a radio-gas chromatograph using the isotope dilution method. In addition the total concentration of the free and bound hydroxy acids which cannot be lactonized, was determined.

## Introduction

Lactones are important flavor components in various natural products. Boldingh and Taylor (1) established the presence in butter of various δ-lactones and also, though in smaller amounts, of γ-lactones. By means of the isotope dilution technique they determined the amounts of δ-octalactone, δ-decalactone, δ-dodecalactone and δ-tetradecalactone. The concentrations of these lactones in butter strongly depend on the time of year and range from about 1 to 40 ppm. Their amounts are increased if butter is heated at 140C, which these authors explained by postulating that butterfat contains esterified 4- and 5-hydroxy acids (bound lactones) in the form of monohydroxyacyl triglycerides.

In a short communication (2) the observations which would substantiate the presence of these compounds in butterfat, are described.

In this paper the quantitative determination of the absolute amounts of "free" lactones as well as those of the "bound" lactones will be described. To check the correctness of the method of analysis, the total amounts of lactones have also been determined. For these determinations the isotope dilution technique (3) has been used.

## Experimental Procedure for the Isolation of Lactones from Butterfat

### Materials and Methods

The solvents diethyl ether, light petroleum (bp 40–60C), iso-octane (ex Shell) and chloroform (ex Merck analytical grade) were all freshly distilled; benzene (ex Merck) was analytical grade and used as such.

The butterfat in a commercial packet of Dutch butter (250 g) was extracted by addition of 250 ml light petroleum and separation of the water and petroleum layers by centrifugation. The petroleum layer was filtered to remove undissolved components and evaporated at low temperature and reduced pressure.

Silica columns (length 30 cm, diameter 2 cm) were used, made from 30 g silicic acid (ex Mallinckrodt, containing 6% physically bound water) and 15 g Hyflo (dried overnight at 120C). Thin-layer plates with a thickness of 0.25 mm were prepared by mixing 60 g silicagel G (ex Macherey and Nagel) with 120 ml water and spreading the slurry on glass plates of

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† Died September 4, 1965.

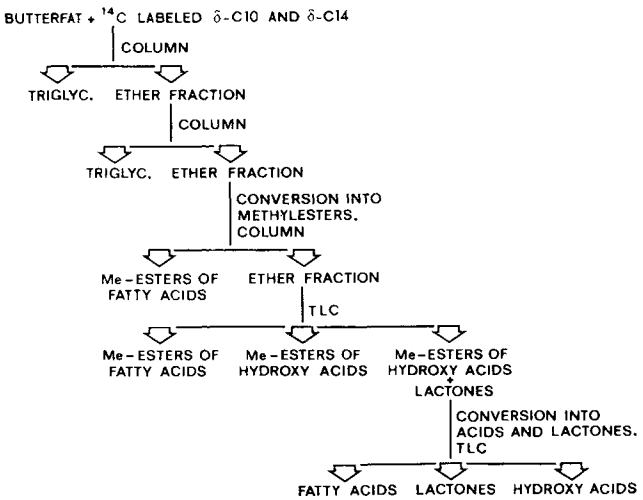


FIG. 1. Scheme of the isolation of total lactone from butterfat.

20 x 20 cm. The plates were activated in a stove for 1 hr at 110C. Silver nitrate plates were prepared in the same way, using a solution of 20 g $AgNO_3$ in 120 ml water.

A stock solution was used containing 100 μg $C^{14}$-labeled δ-decalactone and 235 μg $C^{14}$-labeled δ-tetradecalactone per millititer solution. The purity of these lactones, synthesized by Van Beers and Van der Zijden (3), was checked by means of GLC and was over 99%. The total radioactivity of 1 ml of this stock solution is 196,200 cpm of which 44,640 cpm originate from the δ-decalactone and 151,560 cpm from the δ-tetradecalactone. The radioactivity was determined with a Tri-Carb liquid scintillation counter. For this purpose the samples were dissolved in 10 ml of a solution, containing 4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis {2'-(5'-phenyloxazolyl)} benzene per liter of toluene.

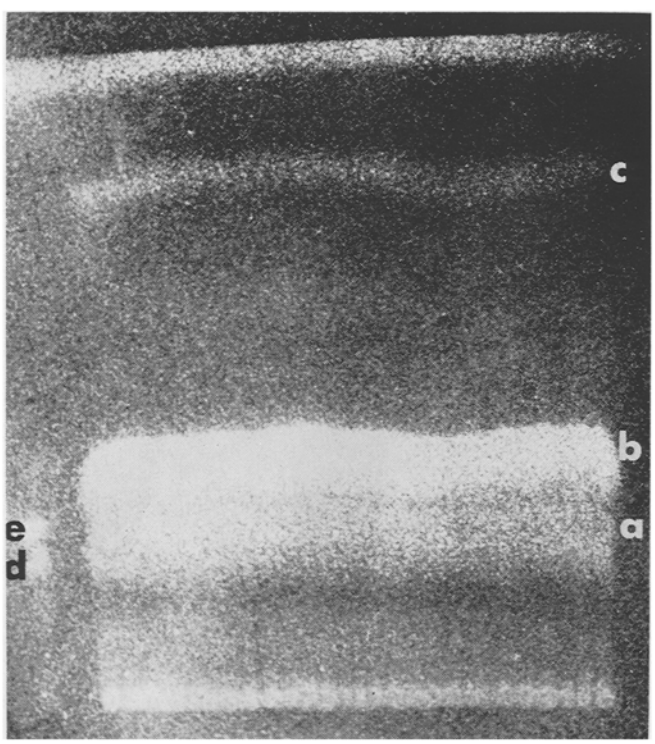


FIG. 2. Separation by TLC of *a*, lactones; *b*, methyl esters of hydroxy acids; *c*, methyl esters of fatty acids. Eluent: iso-octane/diethyl ether 60:40 (v/v). Detection with Ultraphor. References: *d*, δ-dodecalactone and *e*, γ-dodecalactone.

Infrared analysis was carried out on a microscale (5-20μg) by scraping the spots from the chromatoplate and extracting the absorbent at room temperature with chloroform. Silica was removed by centrifuging. The solution was dropped onto 1–2 mg KBr, the chloroform being evaporated in a stream of nitrogen. From the KBr a micropellet (diameter 1.5 mm) was pressed, which was scanned on a Grubb Parsons G.S.4-spectrophotometer provided with a beam condenser. The program used was: gain 13/70; speed 1 μ per 8 min; scanning range 2.5–15 μ.

Gas chromatography was carried out on a Carlo Erba Fractovap with a hydrogen flame ionization detector. The columns (length 200 cm, diameter 4 mm) were packed with 5% PEGA (polyethylene glycol adipate) (column temperature 180C) on Celite (100–120 mesh) or 10% Apiezon L on Diatoport S (80–100 mesh) (column temperature 170C).

The radio-gas chromatograph used is an instrument essentially built according to James and Piper (4) (mass detection: catharometer, radioactivity detection: proportional gas counter). The column (length 120 cm, diameter 4 mm) (column temperature 185C) was packed with 10% PEGA on Celite (60–70 mesh).

**Isolation of Total Lactone from Butterfat**

The isolation-scheme of total lactones from butterfat is shown in Figure 1.

A mixture of 20 g butterfat and 2 ml of the stock solution of δ-lactones is brought onto four silica columns (5 g on each column) after which 4 g of the normal triglycerides is eluted from each column with 300 ml benzene/light petroleum 1 : 1 (v/v). Subsequently, the columns are washed with 300 ml diethyl ether each. The diethyl ether is evaporated at low temperature (< 6C) and reduced pressure, and the residue, dissolved in light petroleum, brought again onto two silica columns. With 300 ml benzene/light petroleum 90 : 10 (v/v) a total amount of 1.6 g normal triglycerides can be eluted. Then the re-

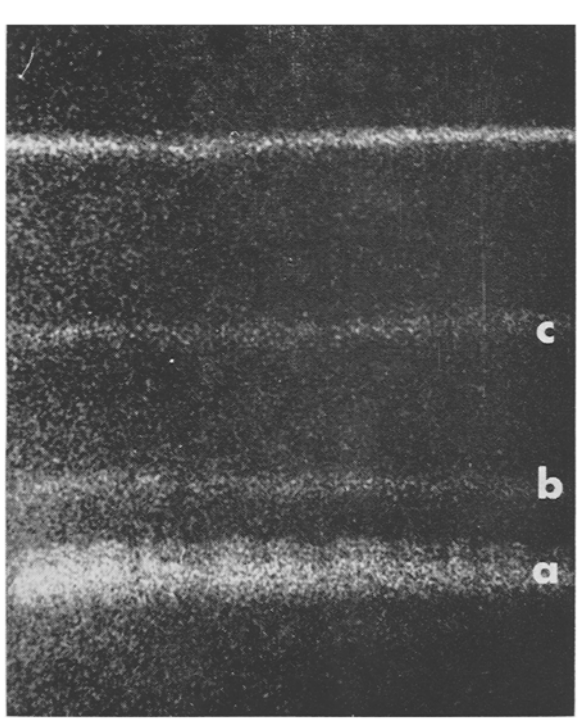


FIG. 3. Separation by TLC of *a*, hydroxy acids; *b*, lactones; *c*, fatty acids. Eluent: iso-octane/diethyl ether 50:50 (v/v). Detection with Ultraphor.

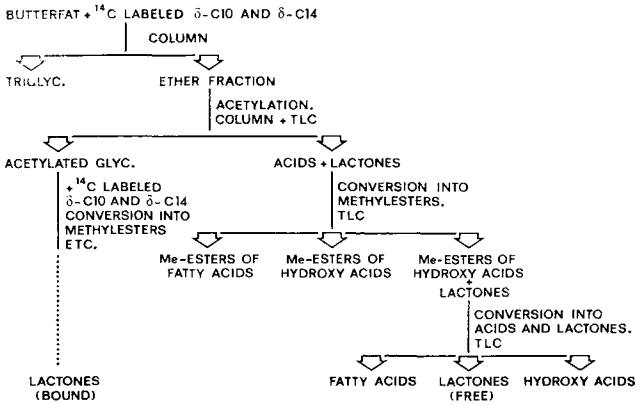


FIG. 4. Scheme of the isolation of "free" and "bound" lactones from butterfat.

maining part is washed out of the columns with 300 ml diethyl ether each.

The diethyl ether of the second column separation is evaporated and the residue saponified with 20 ml 0.15N ethanolic alkali. After saponification the unsaponifiable matter is extracted with light petroleum. Subsequently, the ethanol from the water layer is removed, the solution acidified in the cold with 10 ml 1N HCl containing 10% NaCl, and the acids extracted with diethyl ether. This diethyl ether solution, now containing fatty acids, hydroxy acids and lactones, is washed with water, dried over anhydrous $Na_2SO_4$ and the acids are converted into methyl esters with diazomethane. The diethyl ether is removed, and the concentrate, dissolved in light petroleum, is brought onto a silica column. The greater part of the methyl esters of the fatty acids is eluted with 300 ml benzene/light petroleum 80:20 (v/v). Subsequently the column is washed with 300 ml diethyl ether, the diethyl ether evaporated and the residue brought onto four silica plates. These are developed with iso-octane/diethyl ether 60:40 (v/v), using the ascending technique. After spraying with 1% aqueous Ultraphor (Ultraphor Wt ex Badische Anilin und Soda Fabrik, Ludwigshafen, Germany) solution, three bands are visible in UV-light (see Fig. 2), representing the lactones, methyl esters of

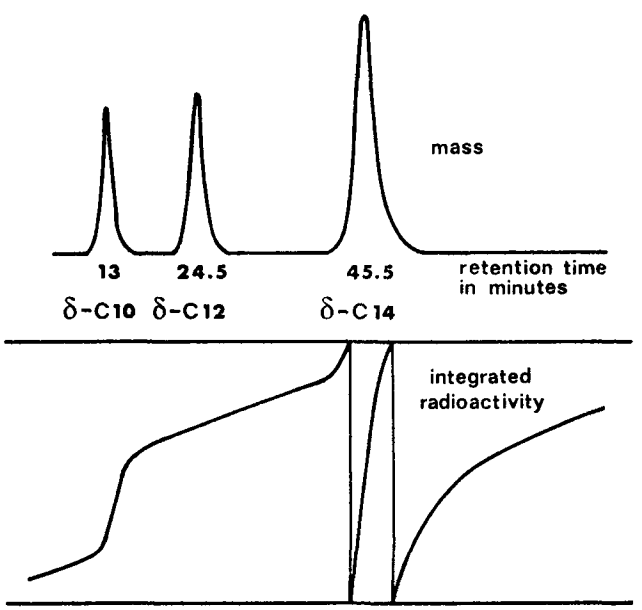


FIG. 5. Analysis of the total lactone fraction from butterfat on the radio-gaschromatograph.

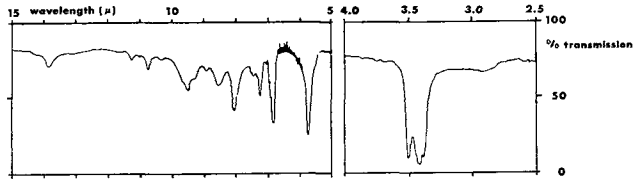


FIG. 6. IR-spectrum of lactones from butter.

hydroxy acids and methyl esters of fatty acids. The fractions are scraped off the plates and the silica is extracted with diethyl ether.

The fraction containing the lactones is saponified with 5 ml 0.15N alcoholic alkali, 10 ml water is added, the ethanol removed and the water acidified in the cold with 5 ml 1N HCl. The acids are extracted with light petroleum, washed mineral acid-free with water containing 10% NaCl and the solution is dried over anhydrous $Na_2SO_4$ overnight to lactonize the 4- and 5-hydroxy acids. Then the solvent is decanted from the $Na_2SO_4$ and, after evaporating the bulk of the solvent, the concentrate is brought onto four silica plates which are developed with iso-octane/diethyl ether 1:1 (v/v). The plates are sprayed with Ultraphor after which in UV-light three bands become visible which indicate hydroxy acids, lactones and fatty acids (Fig. 3). The silica which contains the different fractions, is scraped off the plates and extracted with diethyl ether.

Sometimes the hydroxy acid fraction contains a relatively high amount of "polymerized" lactones. In that case this fraction is saponified again and treated as described previously to separate the lactones from the hydroxy acids.

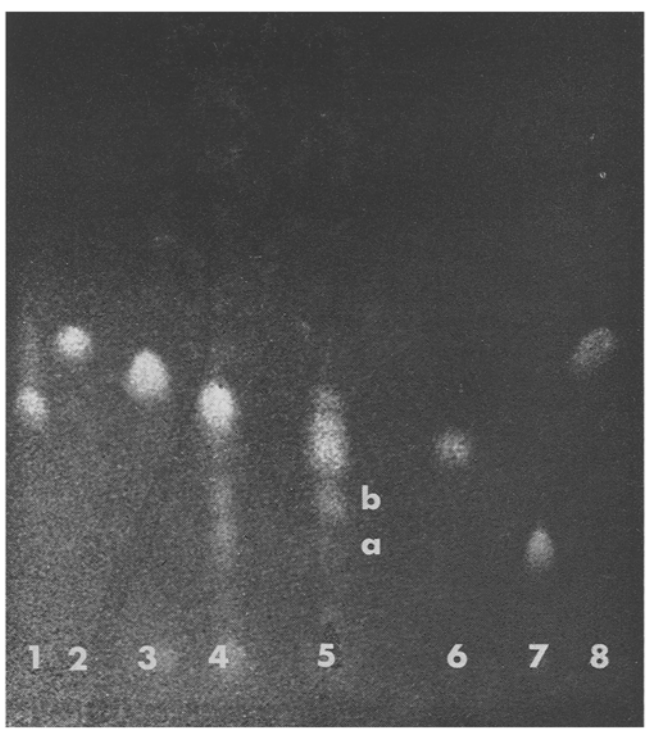


FIG. 7. Separation of lactones and methyl esters of hydroxy acids by TLC on silica impregnated with $AgNO_3$; *1*, δ-dodecalactone; *2*, γ-dodecalactone; *3*, δ-lactone of 2-decenoic acid; *4*, total lactone isolated from butterfat; *5*, methyl esters of hydroxy acids from butterfat; *5a*, the same, containing a *cis* double bond; *5b*, the same, containing a *trans* double bond; *6*, methyl 2-hydroxydecanoate; *7*, methyl 12-hydroxy-9-octadecenoate; *8*, methyl 9-hydroxy-octadecanoate. Eluent: benzene/diethyl ether 80:20 (v/v). Detection with 2,7-dichlorofluorescein.

TABLE I

Amounts of δ-Decalactone and δ-Tetradecalactone in Butterfat in PPM[a]

| Method | Free | | Bound | | Total | |
|---|---|---|---|---|---|---|
| | δ-C10 | δ-C14 | δ-C10 | δ-C14 | δ-C10 | δ-C14 |
| Radio-gaschromatograph | 8 | 21.5 | – | 9.5 | 14.5 | 32.5 |
| Internal standard | 10 | 21.5 | 4.5 | 10.5 | 13.5 | 35.5 |

[a] Rounded off to 0.5 ppm.

### Isolation of "Free" and "Bound" Lactones from Butterfat

The isolation-scheme for "free" and "bound" lactones from butterfat is shown in Figure 4.

For the determination of the composition of the "free" lactones and the esterified 4- and 5-hydroxy acids of butterfat, 1 ml of the stock solution is added to 20 g butterfat. The main part of the triglycerides is removed from the lactones and hydroxy-acid triglycerides by two column separations as described for total lactone. The hydroxy groups of the glycerides (normal diglycerides and monohydroxy-acyl triglycerides) and the sterols of the diethyl ether fraction are acetylated in the cold with acetic anhydride/pyridine 1 : 1 (v/v) (2). After acetylation the sterols and glycerides are separated from the lactones by column chromatography on silica.

The column is eluted first with 200 ml benzene, followed by 100 ml benzene/diethyl ether 95:5 (v/v) and finally with 300 ml diethyl ether. Whereas the first fraction contains glycerides and the third eluate the lactones, the second eluate contains both. This fraction is brought onto a silica plate (eluant iso-octane/diethyl ether 60:40 v/v) to separate glycerides and lactones.

To the combined acetylated glycerides (obtained from the first column fraction and the silica plate by scraping off the silica and extraction with diethyl ether) 2 ml of the stock solution is added. After saponification and removal of the unsaponifiable, the lactones are isolated in the same way as described for total lactone (Fig. 1).

The diethyl ether fraction of the column, to which the lactones obtained from the silica plate are added, is saponified and the unsaponifiable matter is removed. Subsequently, the soap solution is acidified and the acids are extracted with diethyl ether. The acids are converted with diazomethane into methyl esters and brought onto four silica plates. After development with iso-octane/diethyl ether 60:40 (v/v) and spraying with Ultraphor, again three clear bands are visible in UV-light (Fig. 3). Nearly all the radioactivity is found in the lactone fraction, which also contains some methyl esters of hydroxy acids. The lactone fraction is saponified, acidified and the acids are extracted with light petroleum. The light petroleum solution is thoroughly dried over anhydrous $Na_2SO_4$. The solvent is decanted from the $Na_2SO_4$, evaporated and the concentrate is brought onto four silica plates which are developed with iso-octane/diethyl ether 1:1 (v/v). The silica containing the lactones, lying between fatty acids and hydroxy acids, is scraped from the plate and extracted with diethyl ether.

TABLE II

Amounts of Some Saturated Lactones in Butterfat (PPM)

| Lactone | Total lactones | Free llactones | Bound lactones [a] |
|---|---|---|---|
| γ-C10 | 1.2 | 0.6 | |
| δ-C10 | 15.0 | 9.0 | 4.5 |
| γ-C11 | 0.5 | 0.2 | |
| δ-C11 | 0.7 | 0.6 | 0.1 |
| γ-C12 | 1.6 | 1.8 | |
| δ-C12 | 34.5 | 28.5 | 10.8 |
| γ-C13 | 0.5 | 1.5 | |
| δ-C13 | 1.5 | 1.1 | 0.8 |
| γ-C14 | 1.4 | 0.5 | |
| δ-C14 | 34.0 | 21.5 | 10.0 |
| γ-C15 | 1.3 | 3.0 | |
| δ-C15 | 6.4 | 4.0 | 1.6 |
| γ-C16 | 1.3 | 1.1 | |
| δ-C16 | 23.2 | 12.5 | 7.4 |

[a] The amount of γ-lactones is smaller than 0.2 ppm.

### Efficiency of the Isolation

The addition of $C^{14}$-labeled lactones made it possible to follow the isolation of the lactones from the butterfat. In this way the recovery of lactones after the first two column separations was established at about 95–98%. The next steps, saponification of the glycerides, removal of the unsaponifiable matter and conversion of the soap into methyl esters followed by column separation to remove most of the methyl esters of fatty acids, gave a loss of 5% of the radioactivity. The lactone fraction obtained by TLC contained 87% of the radioactivity, whereas the fractions containing methyl esters of fatty acids and of hydroxy acids contained 0 and 1%, respectively. On further separation of this lactone fraction by TLC three fractions were obtained ( see Fig. 3), of which the hydroxy-acid fraction contained 6%, the lactone fraction 66% and the fatty acid fraction 0.3% of the radioactivity. The lactone fraction was again brought onto a silica plate (eluent iso-octane diethyl ether 1:1 v/v). After separation, only one band having the Rf-value of lactones was visible in ultraviolet-light; after extraction from the silica, it contained 60% of the radioactivity.

The principle of the separation of free lactones from the esterified hydroxy acids is based on the fact that glycerides containing a hydroxyl group become less polar after acetylation. So the acetylated glycerides obtained by column chromatography and TLC (see Fig. 4) contained only 2% of the radioactivity.

The yield of the free and of the bound lactones was about 55%.

### Analysis of the Isolated Lactone Fractions

The concentration of the various lactones, both free, bound, and their sum total, was determined by GLC of the isolated lactone fractions. The absolute amounts of the δ-decalactone and the δ-tetradecalactone (further abbreviated as δ-C10 and δ-C14 respectively) were determined by means of the isotope dilution method. To this end the stock solution and the three lactone fractions were analyzed on the radio-gas chromatograph (Fig. 5). The specific activities of δ-C10 and δ-C14 were determined by dividing the measured radioactivity by the areas of the mass peaks. From these specific activities and the amount of $C^{14}$-labeled δ-C10 or δ-C14 added to the butterfat or to the acetylated glycerides, the amounts of free, bound and total δ-C10 and δ-C14 were calculated according to

$$X = P(s_1/s_2 - 1)$$

in which

$X$ = amount of δ-lactone in the butterfat in ppm
$P$ = amount of labeled δ-lactone added in ppm
$s_1$ = specific activity of the labeled lactone added
$s_2$ = specific activity of the lactone isolated

The stock solution was analyzed either immediately before or after the lactone fractions isolated from the butterfat. This was necessary because the sensitivity of the catharometer of the radio-gas chromatograph was to some extent influenced by fluctuations in the ambient temperature.

The concentrations of the free, bound and total

δ-C10 and δ-C14 lactones in butterfat have also been determined in a somewhat different way by analyzing these lactone fractions on the Carlo Erba gas-chromatograph, using 5% PEGA as stationary phase, with and without a second internal standard of δ-C10 and δ-C14 lactone. As an example, the calculation of the total concentration of δ-C10 and δ-C14 lactone is given.

To part of the "total" lactone fraction containing 8585 cpm, a mixture of 10 μg δ-C10 and 23 μg δ-C14 was added. From the GLC analysis with and without the second internal standard the amounts of δ-C10 and δ-C14 lactones were calculated as:

$\delta\text{-C10} + C^{14}\ \delta\text{-C10} = 61.7/(136.7\text{–}61.7) \times 10 = 8.23\ \mu g$
$\delta\text{-C14} + C^{14}\ \delta\text{-C14} = 205/(380\text{–}205) \times 23 = 26.94\ \mu g.$

in which 61.7 and 205 are the areas of the δ-C10 and δ-C14 peaks before adding the second internal standard; 136.7 and 380 the areas of the δ-C10 and δ-C14 peaks after adding the second internal standard. The area of the δ-C12 peak is taken as 100.

The ratio of $C^{14}$ δ-C10 to $C^{14}$ δ-C14 lactone of the total lactone fraction determined by the radio-gas chromatograph is 18.4 to 81.6, so that the radioactivity originating from $C^{14}$ δ-C10 is 18.4/100 x 8585 = 1580 cpm and from $C^{14}$ is 81.6/100 x 8585 = 7005 cpm. This corresponds with 1580 x 100/44,640 = 3.54 μg $C^{14}$ δ-C10 and 7005 x 235/151,560 = 10.60 μg $C^{14}$ δ-C14. The amount of δ-C10 consequently is 8.23–3.54 = 4.69 μg and of δ-C14 26.94–10.60 = 16.34 μg.

Per 1 g butterfat $C^{14}$ δ-C10 and $C^{14}$ δ-C14 were added in amounts corresponding with 4464 and 15,156 cpm. The total concentration of δ-C10 lactones in butterfat is then 4464/1580 x 4.69 = 13.3 ppm and δ-C14 15,156/7005 x 16.34 = 35.4 ppm. In the same way also the concentrations of free and bound δ-C10 and δ-C14 lactones were determined.

The concentrations of the other lactones were deduced from their ratio to δ-C10 or δ-C14, a correction being applied for the relative loss of the lactones shorter than δ-C14.

## Results and Discussion

From the IR-analysis of the isolated lactones (Fig. 6) it was concluded that acids, hydroxy acids and sterols were absent, because no carboxyl C = O (5.85 μ) and OH (2.95 μ) absorptions were found. Comparison with the published spectrum (1) proves that mainly δ-lactones are present. γ-lactones (C = O at 5.68 μ) could not be detected because their amounts are very small in comparison with those of the δ-lactones (C = O at 5.75 μ).

There is a satisfactory agreement between the results obtained by the above-mentioned two methods for the determination of the δ-C10 and δ-C14 lactones (Table I).

The absolute amounts of other saturated γ- and δ-lactones were calculated from the gas chromatographic analyses (using PEGA) obtained with the Carlo Erba. These lactones, mentioned in Table II, were identified by comparing their retention times with those of a synthetic mixture of known saturated γ- and δ-lactones. In addition, the total lactone fraction isolated from butterfat was analyzed gas chromatographically, using Apiezon L as stationary phase, to verify the identification.

Table II gives the amounts of the saturated γ- and δ-lactones from C10 to C16 inclusive. The concentrations of lactones with less than 10C atoms were not calculated from their ratio to δ-C10 because the relative loss of these lactones with respect to δ-C10 is unknown. For the determination of these short-chain lactones it is necessary to have short-chain $C^{14}$-labeled lactones available.

Boldingh and Taylor (1) mentioned the occurrence of unsaturated lactones in butterfat, and they identified an unsaturated δ-lactone of 9-dodecenoic acid and γ-lactone of 6-dodecenoic acid. The occurrence of lactones having a double bond in the side chain was evidenced in the total lactone fraction by TLC on silica impregnated with silver nitrate (Fig. 7, spot 4). The Rf-value of the δ-lactone of 2-decenoic acid (spot 3) is in between that of saturated γ- and δ-lactones, probably due to the position of the double bond.

Indeed, in the gas chromatograms obtained with the Carlo Erba using PEGA as immobile phase, some small peaks occur of which the retention times differ from those of saturated γ- and δ-lactones. Since the sum of the concentrations of these components is smaller than 2 ppm, these unknown, probably unsaturated, lactones are not mentioned in Table II. In the present investigation, however, no attempts have been made to identify unsaturated lactones.

The amounts of "bound" lactones are about one third of the total lactone content in butter, which may be due to the fact that a normal packet of butter was used in the investigation. In fresh butter, the amounts of "bound" lactones are greater and the amounts of free lactones smaller than in older butter (1,3).

Apart from hydroxy acids having the hydroxyl group at the 4- or 5-position, such acids having their hydroxyl group at other positions in the acyl group have also been isolated (Figs. 1 and 4). In a dry solution the 4- and 5-hydroxy acids form γ- and δ-lactones; the hydroxy acids with the hydroxyl group not at the 4- and 5-positions are converted into the corresponding methyl esters with diazomethane. After TLC separation (Fig. 2), methyl esters of hydroxy acid were still present in the fraction containing the lactones (and the radioactivity). After conversion of the methyl esters into acids, the lactones were separated from these hydroxy acids by TLC (Fig. 3). The methyl esters of the hydroxy acids and the hydroxy acids of these two TLC separations were combined. The total amount of these hydroxy acids is 0.12% that of the free hydroxy acids 0.04, and that of the bound hydroxy acids 0.08%. These amounts were determined after conversion of the hydroxy methyl esters (Fig 2b) into tritiated hydroxy methyl esters. The $H^3$-radio-activity was measured and compared with the $H^3$-radio-activity of tritiated methyl stearate used as an internal standard (5).

The composition of these hydroxy acids is too complex to be determined solely by GLC and TLC, because they differ in chain-length and position of the hydroxyl group. On a $AgNO_3$ silica plate the methyl esters of the hydroxy acids could be separated into various fractions (Fig. 7, No. 5). IR-analysis of spots a) and b) (Fig. 7, No. 5) confirmed the occurrence of cis- and trans-unsaturated hydroxy fatty acids.

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