MINOR CONSTITUENTS OF HUMAN MILK (I) IDENTIFICATION OF CYCLOHEXANEUNDECANOIC ACID AND PHYTANIC ACID IN HUMAN MILK FAT BY A COMBINATION GAS CHROMATOGRAPH-MASS SPECTROMETER

H. EGGE, U. MURAWSKI, P. GYÖRGY and F. ZILLIKEN
Department of Physiological Chemistry, University of Marburg, Germany and the Philadelphia General Hospital, Philadelphia, USA

Received 24 December 1968

1. Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) has been first isolated from butterfat [1]. In patients suffering from Refsum's disease (heredopathia atactica polyneuritiformis) phytanic acid is one of the major constituents of serum- and organ-lipids [2–7]. It was also found to occur in low quantities in the serum of healthy persons [8]. From experiments with rats [9–11] and patients with Refsum's disease [12, 13], it has been shown that phythol is transformed to phytanic acid. Therefore phytanic acid may always be present in low quantities after ingestion of chlorophyll. Cyclohexaneundecanoic acid was first isolated from butterfat [14] and sheep perinephric fat [15]. This communication deals with the identification of phytanic acid and cyclohexaneundecanoic acid as normal minor constituents of human milk free fatty acid (FFA) and triglyceride (TG) fractions. Quantitative gas chromatography shows, that phytanic acid is the major portion of the branched chain fatty acid occurring in human milk.

2. Methods

Cream of fresh human milk was thoroughly mixed with 10 volumes/weight of 70% aqueous methanol, pH 8.5. After centrifugation (3000 × *g*; 4°C) most of the FFA remained in the supernatant. The latter was extracted twice with pet. ether (40–60°C). After acidification of the methanolic solution with hydrochloric acid, the FFA were extracted with pet. ether and methylated with diazomethane. The yield was 70–80% of the total FFA present. The fatty acid esters (FAE) were distilled under high vacuum 10^{-4} Torr, 60–120°C, yielding a colourless liquid. Catalytic hydrogenation was effected with PtO_2 in a solution of methanol-methylenchloride (2:1). Saturated straight chain FAE were removed in form of the urea inclusion compound [16]. Transesterification of triglycerides was achieved with 0.1 N potassium hydroxide in methanol/benzene solution (1:1) at 4° within 1–3 hours. Gas chromatography was performed with a Carlo Erba fractovap modell GB with flame ionisation detectors. An LKB 9000 gas chromatograph-mass spectrometer instrument was used for obtaining the mass spectra. The conditions for gas chromatography were the same as given in the legend of fig. 1. The molecule separator was kept at 250°; the mass spectra were obtained at constant accelerating voltage of 3.5 kV with an electron energy of 70 eV; scanning time was about 3 sec in the mass range *m/e* 12–350 [17]. All solvents used were of reagent grade or redistilled.

3. Results

The FFA in human milk fat are a very complex mixture. Fig. 1 shows a section of a gas chromatogram of the FAE after hydrogenation and urea frac-

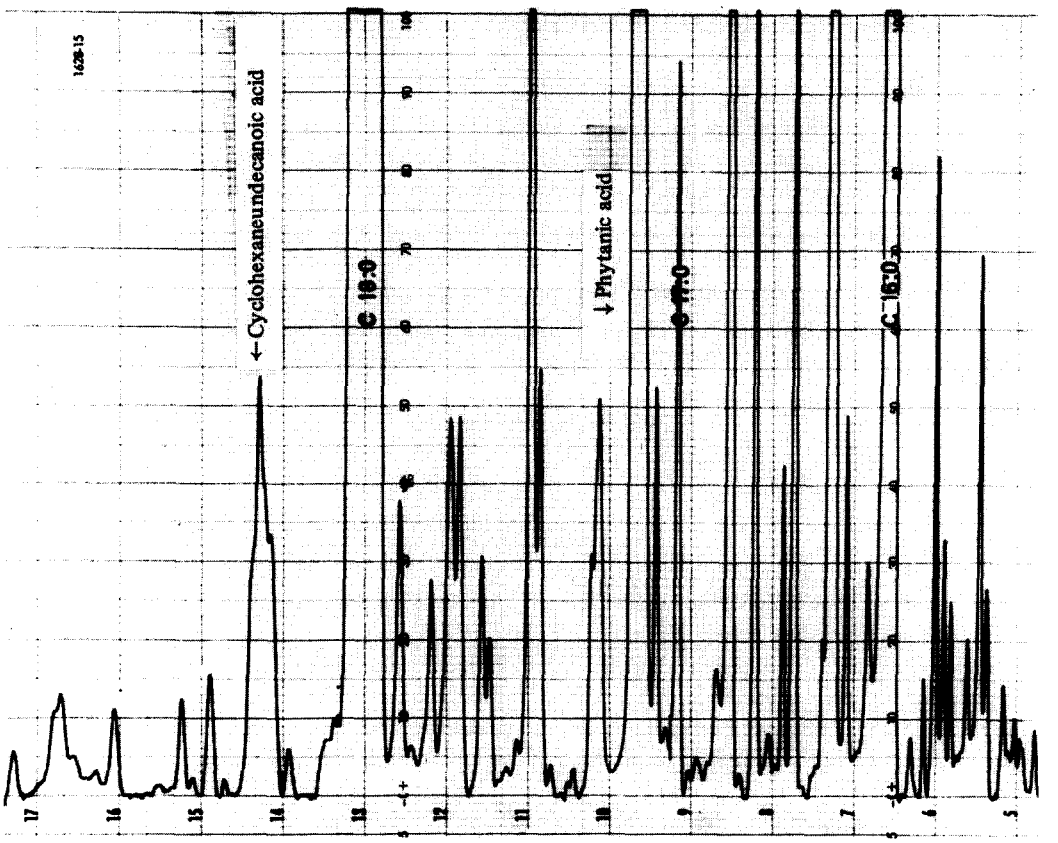

Fig. 1. Gas chromatogram of the FFA-fraction after hydrogenation and urea fractionation; capillary column 50 m × 0.25 mm; stationary phase polyphenylether: temperature program 150–220°; heating rate 1°/min; carrier gas: He; flow rate 1.3 ml/min; sample quantity 0.02 μl, without splitting; injection block temperature 280°; detector FID; measuring range 1/8.

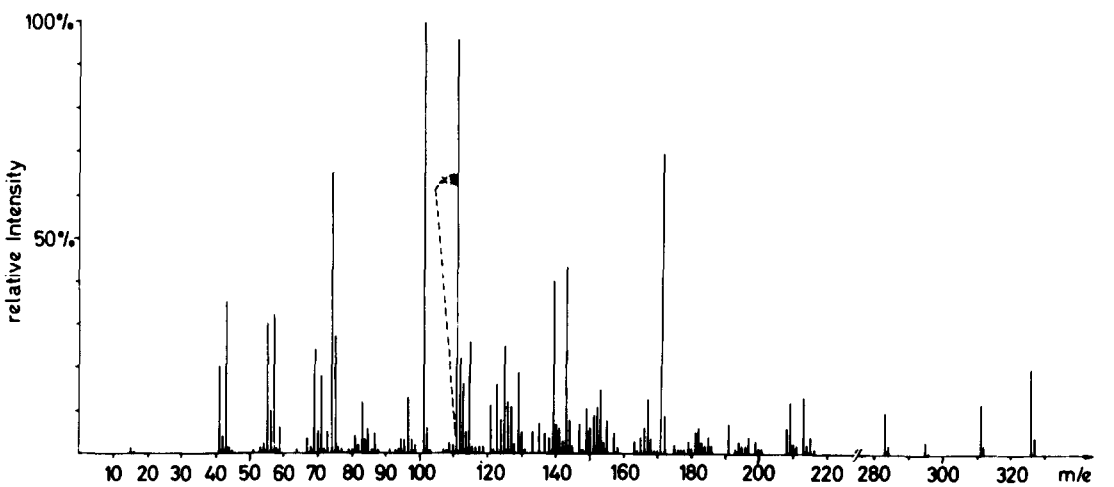

Fig. 2. Phytanic acid methyl ester from human milk.

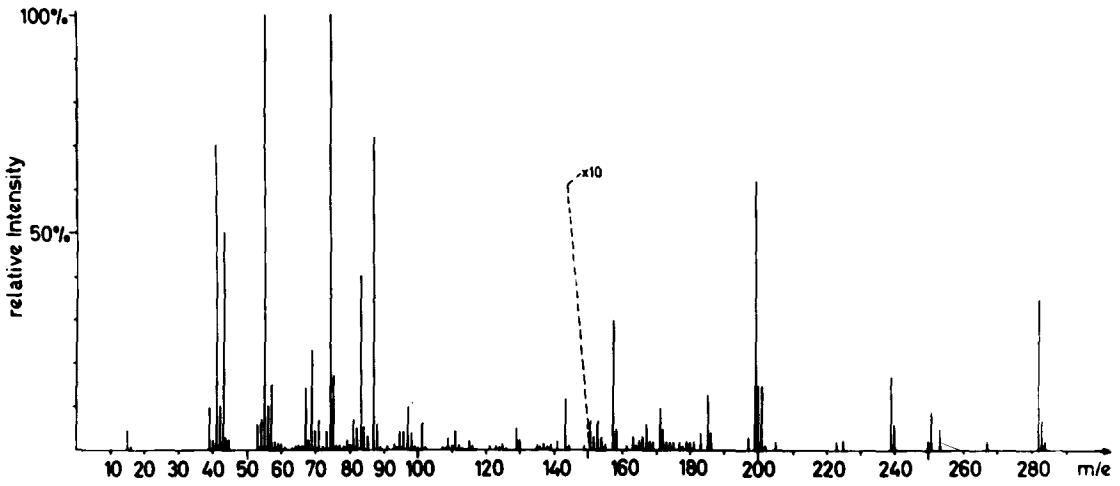

Fig. 3. Cyclohexaneundecanoic acid methyl ester from human milk.

tionation. Phytanic acid methyl ester is the major peak in the C_{17}-region, whereas cyclohexaneundecanoic acid methyl ester is only partly resolved from other accompaning esters. Mass spectra taken at this point also show the presence of di-*n*-butylphthalate, C_{18}-hydroxy acid and branched chain C_{19}-acid [19]. A better gas chromatographic resolution and mass spectrum of cyclohexaneundecanoic acid was obtained using silicon rubber SE 30 as an unpolar liquid phase where the cyclohexylic acid has a retention time corresponding to a carbon number 17.85 [18]. Stearic acid methyl ester was almost completely removed from the sample by repeated urea fractionation. In a quantitative evaluation of the gas chromatograms of the FFA fraction 0.03% of phytanic acid and 0.05% of cyclohexaneundecanoic acid methyl esters were found. This latter value is certainly too high because as can be seen in fig. 1 the resolution is very incomplete at this point and other components may coincide with the cyclohexaneundecanoic acid. Triglycerides of human milk purified by chromatography on silicagel contain essentially the same fatty acids as the FFA. It is, however, noteworthy that together with a higher percentage of saturated fatty acids the amounts of phytanic and cyclohexaneundecanoic acids are also considerably increased.

Acknowledgement

The authors are greatly indebted to Dr. R.Ryhage from the Karolinska Institutet, Stockholm, for the opportunity to run samples on the LKB 9000 instrument in his laboratory.

References

[1] R.P.Hansen and F.B.Shorland, Biochem. J. 50 (1951) 358.
[2] E.Klenk and W.Kahlke, Z. Physiol. Chem. 333 (1963) 133.
[3] W.Kahlke, Klin. Wschr. 42 (1964) 1011.
[4] K.Try, O.Stokke and L.Eldjarn, Scand. J. clin. Lab. Invest. 17 (suppl. 86) (1965) 195.
[5] R.P.Hansen, Biochim. Biophys. Acta 106 (1965) 304.
[6] W.Stewart Alexander, J. Neurol. Neurosurg. Psychiat. 29 (1966) 412.
[7] M.Rake and M.Saunders, J. Neurol. Neurosurg. Psychiat. 29 (1966) 417.
[8] G.J.Kremer, Z. Physiol. Chem. 344 (1966) 227.
[9] G.J.Kremer, Klin. Wschr. 43 (1965) 517.
[10] E.Klenk and G.J.Kremer, Z. physiol. Chem. 343 (1965) 39.
[11] B.Steinberg, J.Avigan, C.Mice and J.Baxter, Biochem. Biophys. Res. Commun. 19 (1965) 412.

[12] W.Stoffel and W.Kahlke, Biochem. Biophys. Res. Commun. 19 (1965) 33.
[13] B.Steinberg, J.Avigan, C.Mice, L.Eldjarn, K.Try and S.Refsum, Biochem. Biophys. Res. Commun. 19 (1965) 783.
[14] J.C.M.Schogt and P.Haverkamp Begemann, J. Lipid Res. 6 (1965) 466.
[15] R.P.Hansen and T.Gerson, J. Sci. Fd. Agric. 18 (1967) 225.
[16] J.Cason, G.Surell, C.F.Allen, G.A.Gillies and S.Elberg, J. Biol. Chem. 205 (1963) 435.
[17] R.Ryhage, J. Dairy Res. 34 (1967) 115.
[18] F.P.Woodford and C.M.Van Gent, J. Lipid Res. 1 (1960) 188.
[19] H.Egge, U.Murawski and F.Zilliken, in preparation.