The C-20 Hydrocarbons of Butterfat

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Hydrocarbons were isolated from butterfat by cold-finger molecular distillation at 35°. The three major hydrocarbons were identified as the C-20 compounds, 3,7,11,15-tetramethylhexadec-1-ene (phyt-1-ene), 3,7,11,15-tetramethylhexadec-2-ene (phyt-2-ene), and 3-methylene-7,11,15-trimethylhexadec-1-ene (neophytadiene). The following hydrocarbons were also shown to be present: 2,6,10-trimethyltridecane, *n*-pentadecane, *n*-hexadecane, 5-methylhexadecane, *n*-heptadecane, *n*-octadecane, and 2,6,10,14-tetramethylhexadecane (phytane). Of these saturated hydrocarbons, *n*-octadecane was the most abundant. The total concentration of the hydrocarbons reported here is of the order of 30 ppm of butterfat. Neophytadiene, but not phyt-1-ene or phyt-2-ene, was also isolated from pasture grass.

Cold-finger molecular distillation of butterfat has been used in the investigation into the compounds responsible for the desirable flavor of butter (Forss *et al.*, 1966, 1967; Stark *et al.*, 1973). The three major classes of compounds thus isolated were acids, lactones, and hydrocarbons. The present paper describes the identification of the major components of the hydrocarbon fraction.

EXPERIMENTAL SECTION

Isolation. Butterfat prepared from sweet-cream butter was subjected to molecular distillation at 35° and the hydrocarbon fraction of the distillate separated from the polar compounds by silicic acid column chromatography as previously described (Stark *et al.*, 1973; Stark and Urbach, 1974).

Preparative Thin-Layer Chromatography. Five thin-layer plates (20 × 20 cm) were prepared from a slurry consisting of 45 g of Aluminium Oxide G (E. Merck AG, Darmstadt) suspended in a solution of 10 g of $AgNO_3$ in 65 ml of H_2O, with the spreader at the 0.25-mm setting. The plates were allowed to dry in air and were then washed twice by chromatographic development with distilled diethyl ether. They were activated for 10 min at 115° and allowed to cool in a desiccator over concentrated H_2SO_4. The total hydrocarbons from 100 g of butterfat were spotted on the starting line of the five plates. Two plates per tank were developed at 0° to the top edge of the plate with 16% distilled diethyl ether in distilled light petroleum, bp 60–80°. No separation was obtained if light petroleum, bp 34°, was used instead of the higher boiling fraction. A spray of 0.1% 2′,7′-dichlorofluorescein in methanol followed by a water spray visualized the hydrocarbons as deep red bands on a pale pink background in visible light and as strongly absorbing bands on a fluorescent background in uv light (365- and 254-nm lamps used simultaneously). No contrast was obtained either in visible or uv light without the water. The bands from the five plates were eluted with diethyl ether for examination by ir and gc–ms.

Gas Chromatography and Mass Spectrometry. The following columns were used for gas chromatography: (1) Carbowax 20M SCOT column, 50 ft × 0.020 in. (Perkin-Elmer Corp., Norwalk, Conn.), (2) 5% Apiezon L on 100–120 mesh Chromosorb G-AW in a glass column (7 ft × 0.075 in. i.d., 0.125 in. o.d.), (3) 5% Carbowax 20M–0.25% isophthalic acid (Clarke and Fredricks, 1967) on 100–120 mesh Chromosorb G-AW either in a Teflon column (30 in. × 0.085 in. i.d., 0.125 in. o.d.) or stainless steel column (4 ft × 0.09 in. i.d., 0.125 in. o.d.), the last being used for combined gc–ms in the apparatus described by Stark *et al.* (1967). Mass spectra were recorded at 70 eV.

Infrared Spectrometry. Ir spectra were obtained in 1 × 5 mm KBr pellets on a Beckman-IR7 infrared spectrophotometer.

Preparative Gas Chromatography. Hydrocarbons from 50 g of butterfat were separated on a 6 ft × 0.172 in. i.d. (0.25 in. o.d.) stainless steel column packed with 12.5% Carbowax 20M–terephthalic acid on 60–80 mesh Chromosorb G-AW DMCS operated isothermally at 125°. The individual hydrocarbons were trapped in U-tubes filled with glass beads cooled in liquid nitrogen, and the hydrocarbons washed out of the traps with distilled light petroleum, bp 34°, which was then evaporated.

$KMnO_4$ Oxidation. The hydrocarbons from the preparative gas chromatography were dissolved in 0.5 ml of acetone, and then 30 mg of $KMnO_4$ was also dissolved in the acetone. A brown precipitate formed quickly. The mixture was allowed to stand overnight at room temperature, and then 15 mg of $Na_2S_2O_5$ was added which immediately decolorized the remaining $KMnO_4$. One milliliter of 10 *N* H_2SO_4 was added to decompose the MnO_2 and the solution was then extracted with three lots of 2 ml of diethyl ether, dried with Na_2SO_4, and reduced to 0.5 ml; the oxidation product was examined by gc–ms.

Phytol (10 μl, E. Merck, AG, Darmstadt) was oxidized in a similar manner.

Hydrogenation. The mixed hydrocarbons were hydrogenated with Adam's catalyst ($Pt_2O{\cdot}H_2O$) and examined before and after hydrogenation on the Carbowax 20M SCOT column. Synthetic phytane was prepared by the hydrogenation of phytol.

Hydrocarbons from Pasture Grass. Pasture grass (73 g dry weight, dried at 65° for 2 days) was extracted in a flame proof Waring Blendor with about 2 l. of distilled light petroleum, bp 34°. The macerated material was filtered through a sintered glass Büchner funnel to give a clear greenish yellow filtrate. The light petroleum was distilled off leaving a light brown shellac-like residue spread over about a 6-in. diameter circle at the bottom of a 5-l. flask. The residue had the odor of hay. Volatile components were collected from the residue by cold-finger molecular distillation at 50° and separated into hydrocarbons and polar compounds on a silicic acid column as for the butterfat distillates. The hydrocarbon fraction was made up to 2 ml with light petroleum and examined by gc–ms and by ir in a KBr pellet.

RESULTS

Hydrocarbons from Butterfat. The three major components of the hydrocarbon fraction, compounds A, B, and C in Figure 1a, had gas chromatographic retention times on Carbowax 20M equivalent to the C-18, hypothetical C-18.5, and C-19 *n*-alkanes, respectively. On hydroge-

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nation, all gave a single peak with the retention time and mass spectrum of authentic phytane (2,6,10,14-tetramethylhexadecane) (Figure 1b).

Mass spectra of compounds A, B, and C (Figures 2, 3, and 4) showed molecular ions at m/e 280, 280, and 278, respectively, suggesting a molecular composition of $C_{20}H_{40}$ for A and B and of $C_{20}H_{38}$ for C. The prominent ions at C-8, C-9, C-13, C-14, and C-18 clearly indicated a branched-chain structure. This was consistent with the fact that the retention times were shorter than those expected for n-C-20 hydrocarbons. This evidence identified compounds A and B as isomeric phytenes and C as a phytadiene or phytyne.

The mass spectra of compounds D and E (Figure 1a) showed molecular ions at m/e 278. Both gave phytane on hydrogenation which identified them as phytadienes or phytynes but the quantities available were too small for complete identification.

R_f values on alumina/$AgNO_3$ thin-layer chromatograms were 0.74, 0.91, and 0.62 for A, B, and C, respectively. Compounds D and E were not detected because they were present in too low a concentration. There was also a band at the solvent front which contained saturated hydrocarbons identified by ir and gc–ms data as 2,6,10-trimethyltridecane, n-pentadecane, n-hexadecane, 5-methylhexadecane, n-heptadecane, n-octadecane, and 2,6,10,14-tetramethylhexadecane (phytane). Of these saturated hydrocarbons n-octadecane was by far the most abundant.

Apart from the usual absorptions for CH_3 and CH_2, ir spectroscopy of compounds A, B, and C showed bands at 737 (isoprene) (Sen Gupta and Peters, 1966) and 1170 cm^{-1} (isopropyl), a doublet at 1367 and 1385 cm^{-1} (isopropyl), but no absorption at 720 cm^{-1} ($(CH_2)_{>3}$). Compound A also absorbed at 908, 994, 1075, 1415, 1640, and 3080 cm^{-1}, all of which are characteristic of a vinyl group. Compound B had an absorption at 1667 cm^{-1} indicative of a trisubstituted ethylene structure (phytol also had this band) and compound C absorbed at 890, 1651 (vinylidene), 902, 990, 3020 (vinyl), and 1595 cm^{-1} (conjugated double bonds).

The only structure for A consistent with the above data was 3,7,11,15-tetramethylhexadec-1-ene (phyt-1-ene) and for C, 3-methylene-7,11,15-trimethylhexadec-1-ene (neophytadiene). Compound B was apparently a 3,7,11,15-tetramethylhexadecene with the double bond adjacent to one of the methyl side chains. The high R_f value of this compound on $AgNO_3$ tlc indicated a weak silver complex characteristic of a sterically hindered double bond (Winstein and Lucas, 1938).

$KMnO_4$ oxidation of compound B yielded a compound whose retention time and mass spectrum were identical with those of the oxidation product of phytol. The mass spectra of both oxidation products were the same as that of 6,10,14-trimethylpentadecan-2-one reported by Ikan *et al.* (1973). Their gas chromatographic retention time was equivalent to that of a hypothetical C-16.5 methyl ketone, consistent with a C-15 methyl ketone with three methyl side chains, based on the assumption that two methyl side chains increase the retention time of a compound by roughly the same amount as one additional carbon atom in the normal chain (Ackman, 1972). Thus, oxidation occurred between C-14 and C-15 and compound B was therefore identified as 3,7,11,15-tetramethylhexadec-2-ene.

Hydrocarbons from Pasture Grass. The major hydrocarbon, compound S, had the same retention time on the Carbowax 20M SCOT column as neophytadiene (Figure 5). There were no peaks corresponding to phyt-1-ene or phyt-2-ene. Ir spectra of the mixture showed absorptions at 737 (isoprene), 890 (vinylidene), 903 and 990 (vinyl), 1170 (isopropyl), 1595 (conjugation), and 1625 and 3100 (vinyl) cm^{-1}. The mass spectrum of compound S was the same as that of the neophytadiene from butterfat, showing that compound S was in fact neophytadiene. The mass spectra of compounds Q and R also had molecular ions at m/e 278 and mass spectra similar to that of neophytadiene suggesting that they were isomeric phytadienes. Their gas chromatographic retention times corresponded to those of compounds D and E from butterfat.

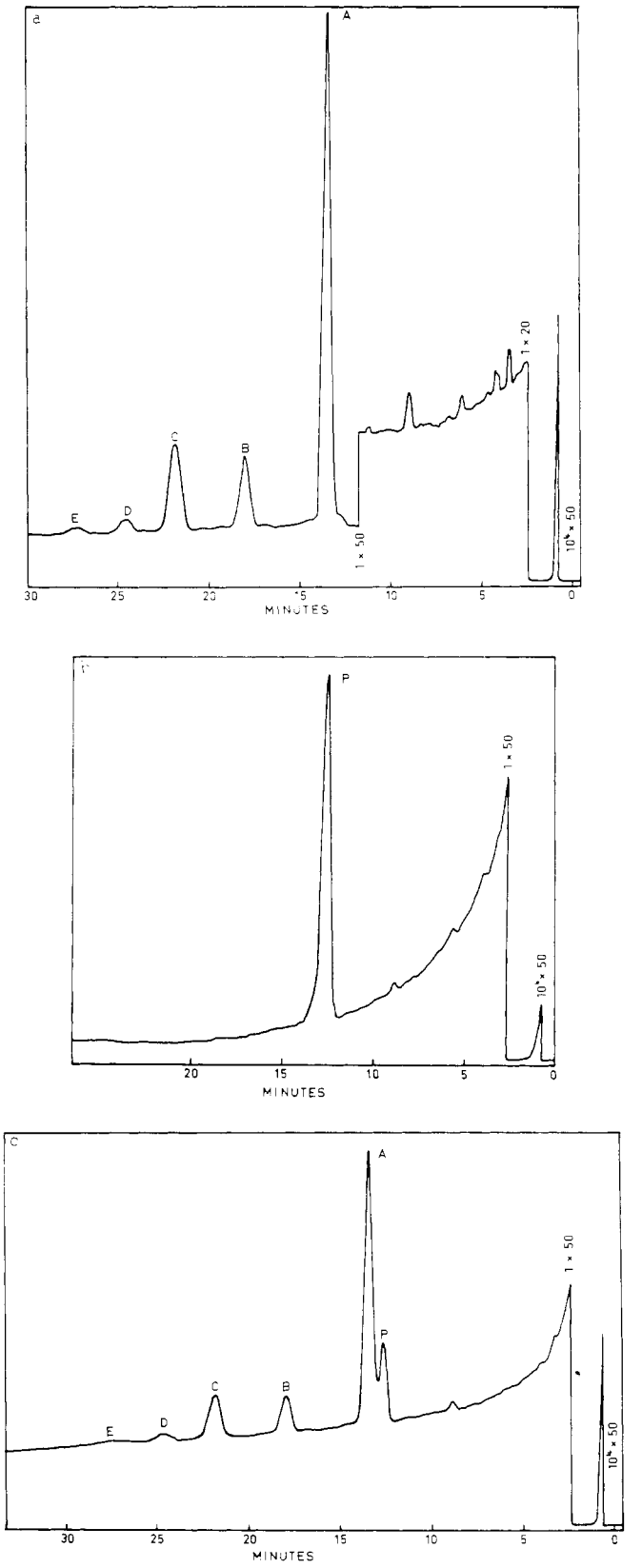

Figure 1. Chromatogram of hydrocarbons from butterfat (a) before hydrogenation; (b) after hydrogenation; (c) mixture of a and b; Carbowax 20M SCOT column; flow rate 3.7 ml of N_2/min; temperature 151° isothermal.

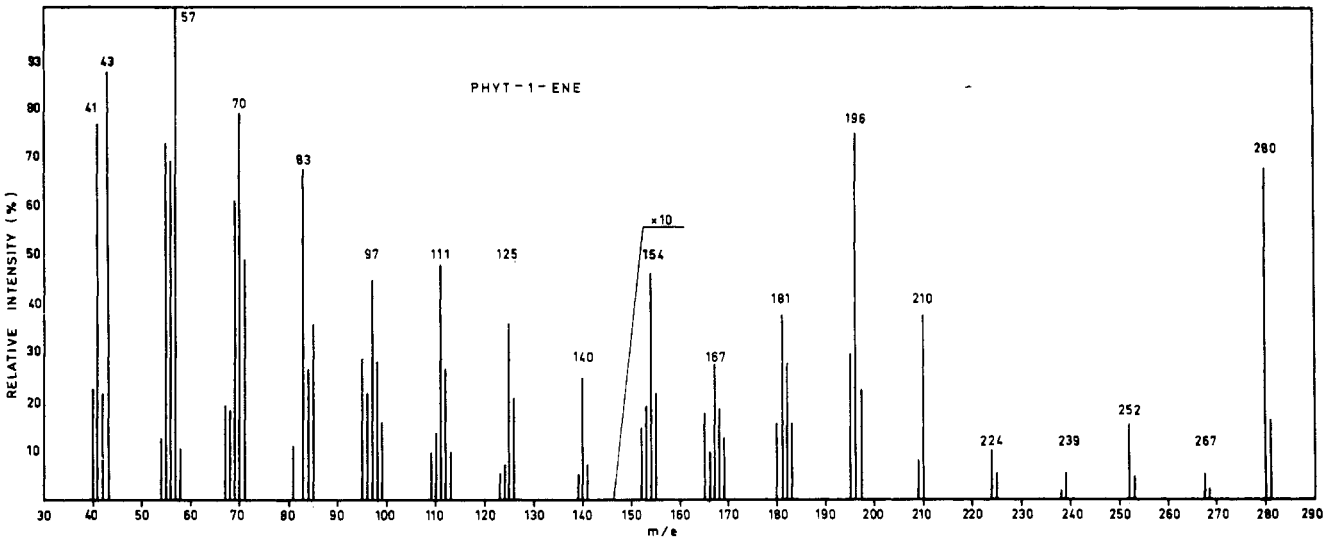

Figure 2. Low-resolution mass spectrum of isolated 3,7,11,15-tetramethylhexadec-1-ene (phyt-1-ene).

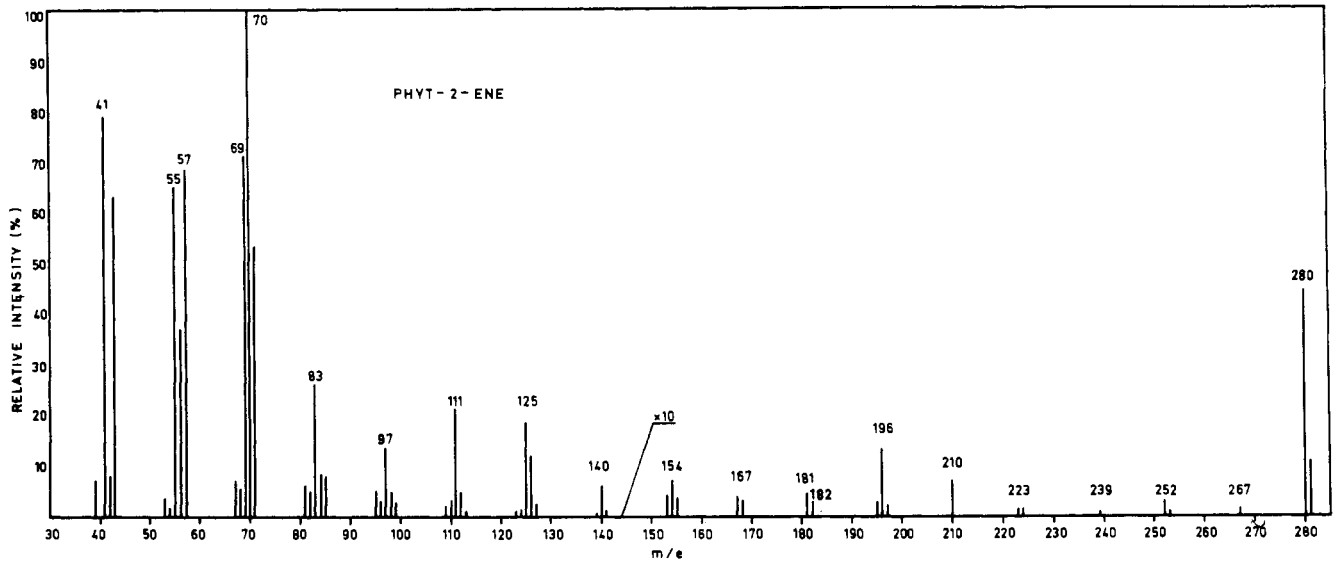

Figure 3. Low-resolution mass spectrum of isolated 3,7,11,15-tetramethylhexadec-2-ene (phyt-2-ene).

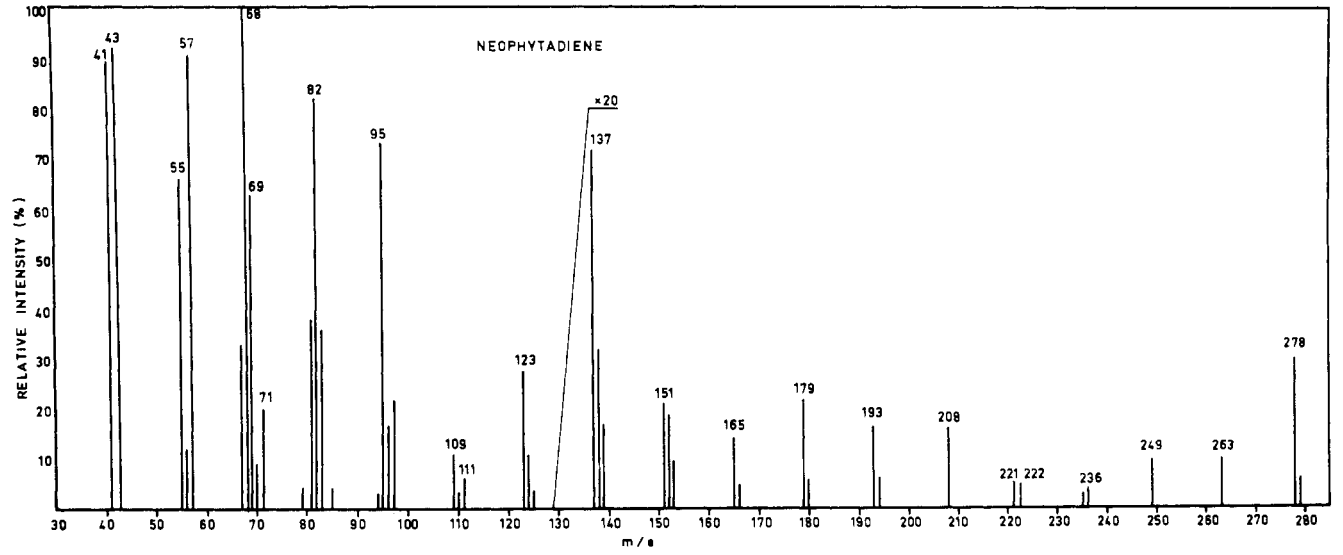

Figure 4. Low-resolution mass spectrum of isolated 3-methylene-7,11,15-trimethylhexadec-1-ene (neophytadiene).

DISCUSSION

$AgNO_3$ Thin-Layer Chromatography. The separating power of a layer, impregnated with $AgNO_3$, can be considerably increased if the development temperature is lowered, as the strength of the $AgNO_3$ π bond increases at lower temperatures (Winstein and Lucas, 1938). At 25°, neophytadiene ($RC(=CH_2)CH=CH_2$) did not separate from phyt-1-ene ($RCH(-CH_3)CH=CH_2$) presumably because the hindered, conjugated methylene double bond did not complex sufficiently with the $AgNO_3$. However, lowering the temperature increased the strength of the complex and good separation was achieved at 0°. This is consistent with the observation of Winstein and Lucas (1938) that both steric hindrance and conjugation reduce π-bond formation.

No useful separation of neophytadiene from phyt-1-ene could be achieved on $AgNO_3$-impregnated silica gel HR (Merck, Darmstadt), either with or without $CaSO_4$ binder; this would seem to indicate that π bonds are more readily formed under alkaline than under acid conditions.

Oxidation. In an attempt to establish the position of the double bond in compound B by the method of Aplin and Coles (1967), the mixture of hydrocarbons was epoxidized with *m*-chloroperbenzoic acid (K&K Laboratories, Inc.) and the mass spectra of the epoxides determined by combined gc–ms. However, the mass spectra of the epoxides did not show the expected features. The mode of fragmentation appeared to be loss of the elements of water followed by breakdown of the resulting acetylenic type fragment. There were no prominent ions corresponding to fragmentation α to the functional group or to transannular fragmentation with concomitant hydrogen transfer. The presence of branching may be the reason for the difference from the results of Aplin and Coles (1967).

The epoxidized mixture was also examined by ir. The bands indicating unsaturation were completely absent and were replaced by absorptions at 835, 875, 890, 937, 1075, 1115, and 1255 cm^{-1}. Epoxidized octadec-1-ene had absorptions at 835, 917, 1128, 1260, 1408, and 3030 cm^{-1}; none of these were present in the unreacted compound.

As epoxidation did not establish the position of the double bond, compound B was oxidized with $KMnO_4$ by a procedure based on that of Baxter and Milne (1969). In preliminary experiments, purified octadec-1-ene was subjected to their oxidation and esterification procedure, and it was shown that although methyl heptadecanoate was the main product, there was also a homologous series of fatty acid methyl esters from C-16 downward. The amount of C-16 and lower acids could be reduced to a ratio for C-17:C-16 ester of 5:1 by carrying out the oxidation at room temperature. Preliminary experiments on the oxidation of compound B indicated that the oxidation produced no long-chain acids. During the oxidation of phytol, acids were deliberately excluded by washing the ether extract with aqueous NaOH. About 3% (with respect to the branched C-18 ketone) of the branched C-13 ketone was also produced in the oxidation of phytol [*m/e* 198 (M^+), 183 (M − 15), 180 (M − 18), 165 (180 − 15), 155 (M − 43), 140 (M − 58), 137, 124, 109 (branching), 95 (branching), 85, 71, 58 (second largest peak), and 43 (base peak)]. A trace of this compound is to be expected from the work of Murray (1959) and probably only indicates branching and not a second isomer of phytol.

Gas Chromatography. Phyt-1-ene, phyt-2-ene, and neophytadiene were separated readily on the polar columns (Carbowax 20M) and phytane had a shorter retention time than phyt-1-ene on the Carbowax 20M SCOT column (Figure 1c).

On nonpolar columns (Apiezon L or SE30) phyt-2-ene and neophytadiene had the same retention times but phytane was clearly separated from phyt-1-ene, the order of elution being phyt-1-ene, phytane, phyt-2-ene. It is usually assumed that on a nonpolar column, monoenes have lower retention times than the saturated analog. However, Bierl *et al.* (1972) also found that in the decene series the dec-2-ene had a slightly longer retention time than decane on SE30, the other isomers having the expected shorter retention times.

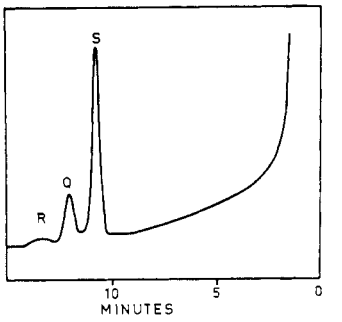

Figure 5. Chromatogram of hydrocarbons from pasture grass: Carbowax 20M SCOT column, flow rate 5.6 ml of N_2/min, temperature 160° isothermal.

Hydrocarbons in Butterfat. Flanagan and Ferretti (1973) reported the identification of phyt-2-ene in milk fat but did not identify phyt-1-ene or neophytadiene. The mass spectra of the two phytenes reported in the present investigation and of Flanagan and Ferretti's (1973) phyt-2-ene are qualitatively similar, including the metastable peaks at *m/e* 61.5, 63.0, 76.3, 88.0, 89.6, 101.5, and 158.0, and it is likely that Flanagan and Ferretti's "compound 280-I" is phyt-1-ene.

In all the butters from pasture-fed cows examined in the present investigation, phyt-1-ene was by far the most abundant of the C-20 hydrocarbons, but the relative amounts of phyt-2-ene and neophytadiene varied, phyt-2-ene sometimes being in excess of neophytadiene whereas on other occasions the reverse was the case. Variations of at least 100% up to a total concentration of 30 ppm were found in the concentration of total C-20 hydrocarbons between different butter samples. This is in agreement with the findings of Ristow and Werner (1968) who found seasonal variations between 25 and 80 ppm of total "olefin" in German butter. Their "Olefin-I" appears to correspond to our phyt-2-ene and the "Olefin-II" fraction is apparently a mixture of phyt-1-ene plus neophytadiene together with higher molecular weight olefins in the winter butter.

The level of C-20 hydrocarbons appeared to be dependent on the diet. When the cows were removed from pasture and fed on a diet of chopped lucerne hay the level of phyt-1-ene and neophytadiene, but not of phyt-2-ene, dropped to about one-tenth of the normal level. The presence of neophytadiene in pasture grass together with the absence of phyt-1-ene and phyt-2-ene from it would suggest that the immediate precursor of phyt-1-ene and neophytadiene in butterfat is not phytol but the neophytadiene of pasture grass. Most of this neophytadiene appears to be hydrogenated to phyt-1-ene in the rumen. In Australia cows are kept on pasture all the year round whereas in the U.S. cows are pasture fed at certain times of the year and stall fed at others. Flanagan and Ferretti (1973) found that phyt-2-ene was the major C-20 hydrocarbon in the American butter which they examined and from our results this indicates that they were analyzing butter from stall fed cows.

It is generally assumed that the tetraisoprene hydrocarbons are biochemically related to phytol. Because phytol is so widespread in nature as part of chlorophyll, we would also expect the C-20 hydrocarbons to be widespread. However, tobacco leaf (Rowland, 1957) and zooplankton (Blumer and Thomas, 1965) are the only reported occurrences of neophytadiene. Phyt-1-ene and

phyt-2-ene have been found only in milk fat. Nagy *et al.* (1969) reported three unidentified $C_{20}H_{40}$ hydrocarbons in bovine liver. It is interesting that they found the fully saturated phytane as the major component of the C-20 hydrocarbon fraction. Kuksis (1964) found unidentified unsaturated hydrocarbons in the C-19 to C-20 region of his chromatograms of the hydrocarbons of seed oils. It therefore seems likely that unsaturated phytane derivatives are much more widely distributed in nature than has hitherto been suspected. Their function is still obscure.

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Comparative Nutritive Value and Amino Acid Content of Different Extractions of Wheat

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The mill fractions obtained by standard milling in a Bühler automatic mill from a single wheat sample, *i.e.* whole wheat flour, bran, white flour (*maida*), and resultant *atta* (fraction left over after removing bran and white flour from whole wheat sample), have been analyzed for their nutritive value, *i.e.*, PER (protein efficiency ratio), NPR (net protein retention), and amino acid analysis. The protein quality index based on PER and NPR at a 10% protein level was found to be highest in resultant atta, followed by bran, whole wheat flour, and white flour. A chemical score based on the essential amino acid content of egg protein and an FAO provisional pattern of milk protein indicates the limiting amino acids in different fractions. The EAAI (essential amino acid index) was also calculated.

In recent years a number of high yielding varieties of cereals such as wheat, rice, and maize have brought a green revolution in the Asian countries, particularly in India where the production has increased by 100% (1960 as basis). It has been reported that the bulk of the Indian population derive 72% of their dietary protein from cereals (Indian Council of Medical Research Special Report, 1953). Evaluation of the protein quality of dwarf rice varieties IR-8 and TN-1 (Taichung Native) and maize using albino rats has been reported by Bressani *et al.* (1971) and Mitra and Das (1971) and Mertz *et al.* (1965) in rice and maize, respectively. Recently Miladi and Hegsted (1972) discovered the RNV (relative nutritive value) and amino acid content of some of the milled fractions of wheat, but no biological test data based on PER, NPR, and amino acid content have been reported so far in different milled fractions used for different baking purposes. The present paper describes results of such work on amber colored dwarf mutant variety Sharbati Sonora developed at the Indian Agricultural Research Institute, New Delhi (Varughese and Swaminathan, 1966).

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MATERIALS AND METHODS

Wheat Samples. Seeds of Sharbati Sonora were collected from a field experiment conducted by the Division of Genetics of the Indian Agricultural Research Institute, New Delhi. In this experiment, nitrogen at the rate of 80 kg/ha was applied to the high nitrogen responsive dwarf variety. P_2O_5 and K_2O were applied at the rate of 40 kg/ha. For chemical analysis samples of whole meal, white flour (60–65%), bran (10–15%), and resultant atta (25–35%) were obtained by grinding wheat in a Bühler automatic mill.

Methods. Samples were dried in a hot air oven at 105° for 6 hr for the determination of moisture. The protein content of the samples was calculated by multiplying the Kjeldahl N by 5.7. The amino acid composition was studied using a Technicon automatic amino acid analyzer. Defatted samples containing 5 mg of protein were hydrolyzed by refluxing with 6 N HCl for 22 hr. After removal of acid by evaporation under reduced pressure, the residue was dissolved in 2 ml of citrate buffer (pH 2.875). An aliquot (0.4 ml) was used for the determination of amino acids according to the method of Moore and Stein (1954). Tryp-