Comparative Deposition of *trans*-10- and *cis*-9-Octadecenoates in Egg and Tissue Lipids of the Laying Hen

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The deposition of [10(11)-^{3}H]-*trans*-10-octadecenoate (10*t*-18:1-^{3}H) was compared to that of [10-^{14}C]-*cis*-9-octadecenoate (9*c*-18:1-^{14}C) in the major egg yolk neutral lipids and phospholipids and in organ lipids from the laying hen. In egg yolk lipids, discrimination occurred against incorporation of 10*t*-18:1-^{3}H for each lipid component and at each acyl position examined except the 1-acyl position of phosphatidylethanolamine. Retroconversion of 10*t*-18:1-^{3}H occurred to the extent that 15 and 27% of the tritium label was associated with 16:1 in yolk triglycerides and total liver lipids, respectively. A second tritiated metabolite, containing 34% of the recovered label in yolk triglycerides, was tentatively identified as a conjugated octadecadienoic acid. Tissue lipid analyses indicated that more 9*c*-18:1-^{14}C than 10*t*-18:1-^{3}H was deposited into all tissues except in the crop/gizzard contents and in feces. These combined data suggest 10*t*-18:1 is preferentially oxidized relative to 9*c*-18:1.

The content of vegetable fat in the Western diet has gradually increased over the last 50 years (Rizek et al., 1983) to the extent that the major source of visible fat is vegetable shortenings and margarines. The unsaturated fatty acids of vegetable oils are normally of the cis configuration. Partial hydrogenation of these oils into shortenings and margarines causes positional and geometrical isomerization of the natural cis acids.

The octadecenoic acid component of these hydrogenated products contains the largest percentage of isomers. These isomeric fatty acids have double bonds from the Δ^5 to Δ^{15} position in the cis configuration and from the Δ^4 to Δ^{16} position in the trans (Elson et al., 1977). Depending upon the catalyst and the extent of hydrogenation, *trans*-octadecenoic isomers account for up to 61% of the monoenoic fat content (24% of total fatty acids) of these commercial products (Parodi, 1976). *trans*-10-Octadecenoic acid (10*t*-18:1), one of the major isomeric acids, averages from 7 to 10% of the total monoenoic fatty acids in various commercial margarines (Parodi, 1976). In salad oils prepared from soybean oil, 10*t*-18:1 constitutes up to 4% of the total fatty acids and 38% of the trans 18:1 (Carpenter et al., 1976).

Our knowledge of the metabolic fate of the isomerized fatty acids comes mainly from feeding studies with hydrogenated fats that contain the entire spectrum of isomers. Two such studies in rats and humans (Wood, 1979; Ohlrogge et al., 1981) have indicated substantially reduced incorporation of the 10*c*-18:1 into various organ lipids compared to the level in the diet. In contrast, 10*t*-18:1 was deposited at only a slightly reduced level. When 10*c*-18:1 was fed to laying hens (Mounts, 1976), discrimination in both yolk triglycerides and phospholipids was evident compared to the deposition of oleic acid.

At this laboratory, the metabolism of individual fatty acid isomers has been studied by means of a dual-isotope-labeling technique in which the ^{3}H-labeled object isomer is fed to laying hens together with a ^{14}C-labeled internal standard fatty acid (Mounts, 1976; Mounts et al., 1971; Lanser and Emken, 1981; Lanser, 1982). The egg from the laying hen serves as a biological trap, and lipid deposited into the egg yolk provides an automatic daily biopsy of ingested fats.

Because 10*t*-18:1 constitutes a large percentage of the *trans*-octadecenoic acid isomers formed during hydrogenation of vegetable oils, and because its content in tissues has only been measured after hydrogenated fat intake, we have examined its specific metabolism by using the dual-labeling technique to compare the deposition of 10*t*- and 9*c*-18:1 into egg yolk and organ lipids of the laying hen. These studies are designed to provide insight into the ability of biological systems to metabolize dietary hydrogenated vegetable oils and to discriminate between geometrical and positional isomers.

MATERIALS AND METHODS

Methyl Esters. Methyl [10-^{14}C]oleate was synthesized via a Wittig coupling and purified as described previously (Lanser, 1982). A mixed Δ^{10} (cis and trans) 18:1 triglyceride, previously prepared at this laboratory (DeJarlais et al., 1982), was the source of 10-octadecenoic acid. The sample was brominated in ether and debrominated with KOH in ethylene glycol. The 10-octadecynoic acid was isolated and converted to the methyl ester with BF_3–MeOH (Morrison and Smith, 1964). Methyl octadec-10-ynoate was reduced by use of tritiated water and Lindlar catalyst (Mounts et al., 1971). Ozonolysis confirmed the product to be methyl [10(11)-^{3}H]-10-octadecenoate. Isomerization to the trans ester was accomplished with *p*-toluenesulfinic acid (Gibson and Strassburger, 1976). The resulting labeled ester mixture was separated by preparative argentation–thin-layer chromatography (Brinkmann plates, 20 cm × 20 cm × 2 mm, silica gel 60 dipped in 20% $AgNO_3$ in ethanol; developing solvent, 20% petroleum ether in benzene).

Radiochromatogram scans (Packard 7201 radiochromatogram scanner) of the *cis*- and *trans*-octadecenoates after separation by argentation–TLC indicated radiochemical purities of 99%.

Purity of the esters was determined by gas chromatography (Perkin-Elmer 3920 GC; 55-m Silar 10C capillary column) to be 99 and 97% for the *trans*-10- and *cis*-9-octadecenoates, respectively.

Experimental Design. Mixtures of fatty acid methyl esters containing 229 μCi of 10*t*-18:1-^{3}H (specific activity 0.03 mCi/mmol) and 228 μCi of 9*c*-18:1-^{14}C (specific activity 1.66 mCi/mmol) (^{3}H/^{14}C = 1.00) were prepared and placed in gelatin capsules for administration as a single dose to each of three laying hens. DeKalb XL hens (51 weeks old) were fed ad libitum a standard laying ration (Purina CF 6501, Ralston Purina, St. Louis, MO). Fatty acid composition of the feed was as follows: 14:0, 0.6%; 16:0, 17.2%; 16:1, 0.8%; 18:0, 5.7%; 18:1, 27.9%; 18:2,

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44.5%; 18:3, 2.9%. Not detectable trans acids (<0.1%) were found. Methods used for administration of radioactive methyl esters, for collection of eggs after feeding, and for lipid extraction have been described previously (Mounts and Dutton, 1976). Lipid analyses were performed on eggs collected during peak incorporation of the radioactive fatty acids. Major neutral lipid and phospholipid components were isolated by preparative TLC under conditions previously described (Lanser and Emken, 1981).

Enzymatic hydrolysis with pancreatic lipase (Mattson and Volpenheim, 1961) and phospholipase A_2 (Robertson and Lands, 1962) was employed to examine positional incorporation into triglycerides and phospholipids.

Radiochemical assays were performed with a Beckman LS-250 liquid scintillation counter with previously described parameters (Mounts and Dutton, 1976). Output from the counter was interfaced to a Mod-Comp computer programmed to calculate disintegrations per minute (dpm) and $^3H/^{14}C$ ratios by means of previously determined quench curves. Selectivity values were calculated as the logarithm of the quotient of the $^3H/^{14}C$ ratio found in the recovered lipids divided by the $^3H/^{14}C$ ratio in the fed mixture.

Radiochromatograms were obtained by counting serially collected column effluent from gas chromatographic (GC) separation (Aerograph gas chromatograph; 8 ft × $^1/_4$ in. aluminum column packed with 15% EGSS-X on 100/120-mesh Gas Chrom P; 190 °C; helium flow 58 mL/min) of lipid methyl esters in scintillation solution. Computer correlation of the inactive GC curve and the radiochemical data provided data identifying the fatty esters containing radioisotope labels (Mounts and Dutton, 1976).

In a second feeding study of the 10*t*- and 9*c*-18:1 fatty esters, 81.9 μCi of 9*c*-18:1-^{14}C and 89.4 μCi of 10*t*-18:1-^3H supplemented with 186.2 mg (628 μmol) of 10*t*-18:1-13,14-d_2 [prepared at our laboratory, 99% pure by GC procedures (DeJarlais et al., 1982); effective specific activity of 10*t*-18:1-^3H was 0.13 μCi/μmol] were administered in two gelatin capsules ($^3H/^{14}C$ = 1.09) to one DeKalb XL hen in lay to compare uptake and deposition of these fatty acids into organ lipids. The 108-week-old hen had been maintained on the same feed as described previously but had been fasted for 17 h. The hen was decapitated after $6^1/_2$ h. The blood was collected, and organs were quickly removed and weighed. The contents of the crop, gizzard, and intestine were analyzed instead of the organ tissue itself. Developing eggs in the ovary and the egg in the oviduct were combined as the "ovary".

Tissue samples were frozen in 2-propanol/dry ice and stored at −20 °C. Lipids were extracted with 3/2 hexane/2-propanol by the method of Hara and Radin (1978) after tissue disruption with a Polytron homogenizer. The lipid extract was washed with aqueous Na_2SO_4.

Methyl esters of tissue lipids were prepared with 5% HCl/MeOH and purified by preparative TLC (Brinkmann plates, 20 cm × 20 cm × 2 mm, silica gel 60; solvent, 1/1/0.05 petroleum ether/diethyl ether/acetic acid). Radioactivity was measured and $^3H/^{14}C$ ratios and selectivity values were calculated.

RESULTS AND DISCUSSION

The experimental design used in this study employed a dual-isotope-labeling technique in which two radioisotope-labeled fatty acids were fed simultaneously to laying hens. The dual-labeling technique allows an experimental fatty acid to be administered along with a fatty acid that acts as an internal standard in an experimental animal. One advantage of this technique is that the subject acts as its own control in that both fatty acids are subjected to the same metabolic environment, thus eliminating variable experimental parameters such as diet and within-subject biological variations. Another advantage is that this technique allows comparison of the metabolism of the labeled fatty acids, without confusion with endogenous or other exogenous fatty acids, by noting differences in isotopic ratios from those in the fed mixture (Sgoutas et al., 1965; Ono and Fredrickson, 1964). Feeding the same internal standard fatty acid in experiments with different experimental fatty acids also allows direct comparison of the isotopic ratios of each of these experimental fatty acids. The calculation of selectivity values, as defined earlier, allows easy recognition of preferential incorporation of the isomers. Positive (negative) values indicate selective incorporation (discrimination against) of the tritiated molecule into lipid fractions.

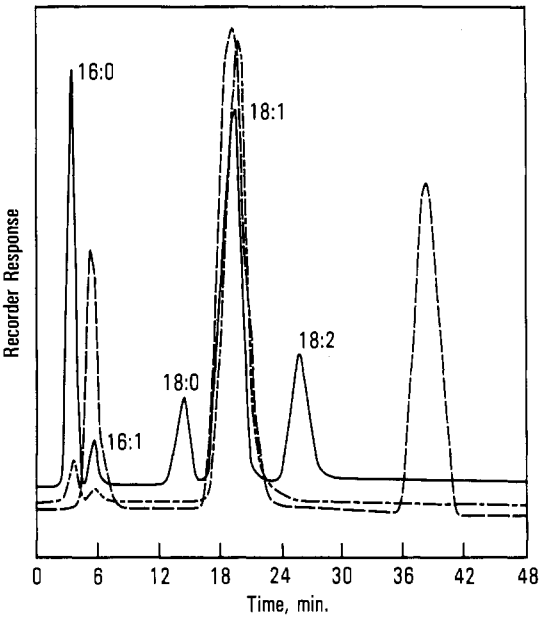

Figure 1. Radio-gas chromatogram of triglyceride methyl esters isolated from egg yolk lipids after feeding 10*t*-18:1-^3H and 9*c*-18:1-^{14}C. Methyl esters were serially collected in scintillation solution from column effluent during gas chromatography. Conditions: 8 ft × $^1/_4$ in. aluminum column packed with 15% EGSS-X on Gas Chrom P, 100/120 mesh; 190 °C; 58 mL/min helium flow. Chromatograms for carbon-14 (-·-) and tritium (--) are correlated with the standard GC tract (—) by computer. Methyl esters are identified by chain length and number of double bonds (e.g. 18:2, 18 carbons with 2 double bonds).

Two results of radio-gas chromatography (Figure 1) of yolk lipid methyl esters were unexpected.

Chain Shortening. The first was that retroconversion, or chain shortening, had occurred with the 10*t*-18:1 isomer to the extent that up to 15% of the tritium label was associated with methyl hexadecenoate (presumably 8*t*-16:1 via a single β oxidation of the trans isomer). Phosphatidylethanolamines (PE), phosphatidylcholines (PC), and triglycerides (TG) contained 11, 15, and 13%, respectively, of the ^3H label recovered in the methyl esters. Apparently very little retroconversion of the cis fatty acid occurred because almost no ^{14}C was detected with the 16:1 peak.

Retroconversion of *trans*-octadecenoates has been shown to occur in the rat, hen, and human. The 8*t*-16:1 isomer was the predominant *trans*-hexadecenoate in rat tissue TG examined after feeding partially hydrogenated safflower oil fatty acids (Ohlrogge et al., 1981). In earlier feeding studies, egg yolk TG contained 8 and 22% of the recovered tritium after feeding ^3H-labeled 12*t*- and 8*t*-18:1 isomers, respectively (Lanser and Emken, 1981; Lanser, 1982).

Table I. Radiochemical Analysis of Egg Yolk Lipids after Feeding 10*t*-18:1-^{3}H and 9*c*-18:1-^{14}C (^{3}H/^{14}C = 1.00)

lipid comp[a]	lipid acyl posn	specific act.[b] ^{3}H	specific act.[b] ^{14}C	^{3}H/^{14}C in yolk lipids	selectivity
CE		487	1242	0.36 ± 0.09 (9)[c]	−0.44
TG		3560	6925	0.51 ± 0.05 (9)	−0.29
	TG 1 + 3	4068	8144	0.56 ± 0.09 (9)	−0.25
	TG 2	2991	7753	0.39 ± 0.03 (9)	−0.41
PE		2158	2676	0.90 ± 0.15 (9)	−0.05
	PE 1	3194	1972	1.54 ± 0.32 (10)	0.19
	PE 2	797	3460	0.24 ± 0.03 (10)	−0.62
PC		2143	6281	0.37 ± 0.04 (9)	−0.43
	PC 1	3146	3526	0.97 ± 0.09 (7)	−0.01
	PC 2	1550	13877	0.15 ± 0.02 (10)	−0.82

[a] Abbreviations: CE = cholesteryl esters; TG = triglycerides; PE = phosphatidylethanolamines; PC = phosphatidylcholines. [b] ^{3}H or ^{14}C dpm/mg of methyl octadecenoate. [c] Average ± standard error determined on egg yolk samples from three hens. The number of samples analyzed is given in parentheses and includes at least one egg yolk from each hen collected during peak incorporation of radioactivity.

Human plasma TG contained from 2 to 6 times more 16:1 from 10*t*- and 10*c*-18:1 than from 9*c*-18:1 (Emken et al., 1985). Since animals do not normally synthesize trans fatty acids, except as metabolic intermediates, the diet must be the source of the *trans*-hexadecenoates or of their precursors.

The isotope labeling used in this and other studies confirms the fact that the *trans*-hexadecenoates must derive from the *trans*-octadecenoates via chain shortening resulting from a single β oxidation. The reason for cessation of the chain shortening is not known. No labeled shorter-chain fatty acids were detected.

Desaturation. The second unexpected result was the presence of a tritiated octadecadienoic acid metabolite that had not been detected in previous feedings of trans acids to laying hens. This metabolite contained up to 34% of the ^{3}H label recovered in yolk TG methyl esters and about 10% in both PE and PC. The peak in the tritium chromatogram had a retention time on a packed EGSS-X gas chromatography column (Figure 1) beyond methyl 18:2 (linoleate) and in the vicinity of methyl 18:3 (linolenate).

Mahfouz et al. (1980) described the desaturation of trans 18:1 acids in rat liver microsomes and found that less than 1% of 10*t*-18:1 was converted to diene. Position of the double bonds was not investigated. Pollard et al. (1980) reported that 15% of 10*t*-18:1 was desaturated by Δ^6- and Δ^5-desaturases. Gurr et al. (1972) provided evidence that the hen's liver is capable of desaturating a 12*c*-18:1 fatty acid at the Δ^9 position to yield linoleic acid.

Our preliminary attempts at identification of this compound suggest another mechanism or desaturase may be involved. GC retention on polar (EGSS-X) and nonpolar (OV-101) columns indicated an 18-carbon chain length. UV showed an absorption at ca. 233 nm. IR analysis had two bands at 10.19 and 10.56 μm, indicating cis,trans-conjugated double bonds. Mass spectra gave a parent ion at m/e 296 resulting from methyl octadecadienoate-d_2. These data suggest the tritiated unknown is a conjugated octadecadienoate. Results of reductive ozonolysis have been inconclusive for locating the double-bond positions because of insufficient sample size.

Yolk Lipids. The patterns for incorporation of the 10*t*-18:1 isomer into lipid classes and acyl positions were generally the same as seen in prior feeding studies with *trans*-octadecenoates with the double bond at even-numbered carbons (Lanser and Emken, 1981; Lanser, 1982). As reflected by negative selectivity values (Table I), the incorporation of 10*t*-18:1 into each of the major lipid fractions and at each position except the 1-acyl position of PE was less than 9*c*-18:1. Cholesteryl esters (CE) and PC displayed the largest discrimination against 10*t*-18:1, TG were intermediate, and PE showed little discrimination.

Table II. Radiochemical Analysis of Tissue Lipids after Feeding 10*t*-18:1-^{3}H and 9*c*-18:1-^{14}C (^{3}H/^{14}C = 1.09)

tissue	specific act.[a] ^{3}H	specific act.[a] ^{14}C	^{3}H/^{14}C ratio	selectivity
crop/gizzard[b]	6294.0	4982.3	1.27	0.06
intestine[b]	1697.2	1903.0	0.89	−0.09
blood	3037.1	5861.6	0.52	−0.32
liver	2176.6	4076.6	0.53	−0.31
ovary	147.7	279.9	0.52	−0.32
heart	1051.4	2740.2	0.38	−0.45
adipose	23.6	29.5	0.80	−0.13
feces	19248.8	13579.4	1.41	0.12
kidney	317.2	1413.3	0.22	−0.69

[a] Total ^{3}H + ^{14}C dpm/mg of methyl octadecenoate. [b] Contents only.

Triglycerides. Large preferential incorporation of 9*c*-18:1 over 10*t*-18:1 was shown in total TG and also in the combined 1- and 3-acyl positions. Discrimination against 10*t*-18:1 was even more pronounced at the 2-acyl position. Similar selective incorporation of a cis fatty acid over a trans acid was also reported in perfused chicken liver (Bickerstaffe and Annison, 1970). Rats maintained on a diet containing partially hydrogenated safflower oil fatty acids had a cis/trans ratio of 2/1 in plasma TG octadecenoates even though the dietary 18:1 content was two-thirds trans (Wood et al., 1977). However, the 10-trans isomer represented the largest percentage of trans 18:1 in plasma TG lipids. Human plasma TG had a selectivity of −0.06 when 10*t*-18:1 was fed simultaneously with 9*c*-18:1 (Emken et al., 1980).

The negative selectivity values determined at the combined 1- and 3-acyl positions and at the 2-acyl position agree with previous acyl positional analyses of TG with rats (Duncan and Garton, 1967, 1969) and with the results of our previous feedings of 8*t*- and 12*t*-18:1 to laying hens (Lanser and Emken, 1981; Lanser, 1982).

Cholesteryl Esters. Cholesteryl esters displayed preferential incorporation of 9*c*-18:1 over 10*t*-18:1, similar to that reported with 8*t*-18:1 (Lanser and Emken, 1981) but opposite to the selectivity shown for 12*t*-18:1 (Lanser, 1982). The positive selectivity for the latter isomer may be due to having a double bond in the important Δ^{12} position. This selectivity for cis fatty acids over trans acids has also been demonstrated in cholesteryl esters from rat liver microsomes (Sgoutas, 1970) and human blood lipids (Emken et al., 1980; 1976). Sgoutas et al. (1976) measured a decrease of nearly 70% in the ^{3}H/^{14}C ratio determined in the incubated mixture as compared with that recovered in CE. Acyl CoA:cholesteryl-*O*-acyl transferase from rat liver microsomes esterified 9*c*-18:1 to cholesterol at 3 times the rate of 9*t*-18:1. Human plasma CE had a selectivity value of −0.54 when 10*t*- and 9*c*-18:1 were fed (Emken et al., 1985).

The enzyme lecithin:cholesterol acyltransferase provides another source of fatty acid for esterification to cholesterol. Because the 2-acyl position of PC contains little 10*t*-18:1, it cannot supply this trans isomer and thus would enhance the negative selectivity in CE.

Negative selectivity values determined on CE components indicate either that preferential incorporation of the 9*c*-18:1 isomer occurs or that selective β oxidation of 10*t*-18:1 occurs. Since data from Ohlrogge et al. (1981) and

Wood (1979) showed no drastic discrimination against this isomer in human or rat tissues, selective β oxidation is a likely explanation and is supported by the negative selectivity values found for all tissue lipids (Table II).

Phospholipids. PC had the most negative selectivity value. PC-2 showed the largest negative selectivity, indicating that the 10*t*-18:1 isomer is largely excluded from this acyl position. The selectivity value at PC-1, however, suggests equal incorporation of the two experimental fatty acids. The same trend was observed in human plasma PC (Emken et al., 1985), except that 10*t*-18:1 was incorporated at PC-1 to the extent that the selectivity value was 0.34. Values for PC and PC-2 were negative.

PE had a small negative selectivity indicating essentially equal incorporation of the cis and trans isomers into this molecule. PE-2 had a large negative selectivity, whereas PE-1 had the only positive value of the egg lipid fractions studied. Similar trends were observed in these phospholipid fractions when 8*t*- and 12*t*-18:1 were fed to laying hens (Lanser and Emken, 1981; Lanser, 1982). PE contains a larger concentration of polyunsaturated fatty acids at the 2-acyl position than PC, and, therefore, the concentration of 9*c*-18:1 is reduced, as evidenced by the difference in specific activity of ^{14}C-containing fatty acids at these acyl positions.

These data follow the general pattern of fatty acid incorporation into phospholipids: that is, that the 2-acyl position is occupied with the more unsaturated fatty acid and the 1-acyl position is esterified with saturated or less unsaturated fatty acids. Obviously, enzymes responsible for esterifying fatty acids to the phospholipids are sensitive to structural differences in the fatty acids. Spatial configuration of trans acids more closely resembles saturated rather than cis-double-bond-containing fatty acids.

Selectivity values for 10*t*-18:1 at PC-2 and PE-2 were intermediate between those previously determined for 8*t*-18:1 and 12*t*-18:1 in yolk lipids (Lanser and Emken, 1981; Lanser, 1982). At PE-1 and PC-1, the 10-trans isomer had the smallest selectivity of the even-numbered trans isomers.

Specific activity data show that there was a larger incorporation of 9*c*-18:1 into PC than PE, and especially into PC-2. Equal ^{3}H activity was measured in total PE and PC and at PE-1 and PC-1, but PC-1 contained about 2 times the ^{14}C acitivity of PE-1. Evidence for the overwhelming selectivity for 9*c*-18:1 at PC-2 is seen in the large specific activity determined at this acyl position. These data account for the difference in selectivity values for PE and PC.

Tissue Lipids. Specific activities (dpm/mg of methyl octadecenoate) and selectivity values for tissue lipids are summarized in Table II and indicate that the fatty acids were being absorbed, metabolized by the liver, and transported throughout the hen. The selectivity value calculated for the crop/gizzard contents was nearly zero (0.06), indicating no discrimination against either isomer during transport to the intestine. All other selectivity values determined for extracted tissue lipids were negative except for the feces (0.12). Kidney tissue showed the largest discrimination against 10*t*-18:1 (−0.69). The selectivity value calculated for ovary lipids (−0.32) closely agreed with the value determined for yolk triglycerides (−0.29), which constitute 70% of the total yolk lipids. The specific activity was low in adipose tissue, suggesting that little of the fed isomers had been stored.

The selectivity value determined for fecal lipids suggests that 10*t*-18:1 may not be as readily absorbed from the intestine of the hen as 9*c*-18:1. Because similar results were not seen in organ studies when 8*t*-18:1 was administered to the laying hen, it may be that the large supplement of unlabeled 10*t*-18:1 in this experiment resulted in some fatty acids (of which 10*t*-18:1 would be a major component) passing through the intestine to the feces. The selectivity value calculated for the intestinal contents (−0.09) also suggests that the labeled 10*t*-18:1 isomer was being lost from the intestinal tract by excretion or was being absorbed into the body faster than 9*c*-18:1. The former explanation seems more feasible. Even if we assume the isotopic ratio determined for the intestinal contents to be the ratio of the fatty acids absorbed, all other "adjusted" selectivity values determined in the various tissues would be less negative but would nevertheless show preferential deposition of 9*c*-18:1 over 10*t*-18:1.

Radio-gas chromatograms of methyl esters from liver total lipids show the same two tritiated metabolites as seen in yolk lipids. Relative percentages vary, however, from those of yolk lipids in that 16:1-^{3}H and conjugated 18:2-^{3}H (proposed) contain 27 and 7%, respectively, of the recovered tritium label.

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Registry No. (*E*)-$HO_2C(CH_2)_8CH{=}CH(CH_2)_6CH_3$, 5684-82-2; (*Z*)-$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$, 112-80-1; hexadecenoic acid, 25447-95-4; cholesterol *trans*-10-octadecenoate, 106621-41-4; cholesterol *cis*-9-octadecenoate, 303-43-5.

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Quantitative Analysis of Aroma Compounds in Wheat and Rye Bread Crusts Using a Stable Isotope Dilution Assay

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A stable isotope dilution assay was developed for the quantitative analysis of acetylpyrazine, 2-methyl-3-ethylpyrazine, 5-methyl-5*H*-cyclopenta[*b*]pyrazine, and 2-acetyl-1-pyrroline in bread crusts. Model experiments showed that these compounds, which contribute significantly to the crust flavor, can be determined with high sensitivity and accuracy. The method revealed that the levels of the three pyrazines lie in similar concentration ranges in the wheat and rye crusts. In contrast, 2-acetyl-1-pyrroline appeared at an approximately 20-fold higher concentration in wheat crust than in rye. Comparison of two different processes for rye bread revealed that the level of the four compounds was twice as high in the three-stage sourdough than in the one-stage process.

During heating of a foodstuff a multiplicity of volatiles is formed in very low concentrations. The aroma compounds that are characteristic for the distinct flavor notes of a food are only small fractions of this volatile mixture. Therefore, they have to be enriched by several concentration steps for quantitative analysis, and the losses caused by these manipulations are corrected with internal standards. However, this method provides only accurate data when the stability and the physical properties of both the compound to be estimated and of the internal standard are very similar. The best internal standard would thus be the compound labeled with an appropriate isotope, provided that the label is not changed during the analytical procedure. The use of a stable isotope labeled internal standard for quantitative analysis was at first reported by Sweeley et al. (1966). After addition of deuteriated glucose as internal standard, they quantified this substance by determination of the ratio of the protium and deuterium forms with the aid of combined gas chromatography–mass spectrometry. In the meantime this method, known as the "isotope dilution assay", has been widely applied for the quantitative analysis of trace components.

We have used an isotope dilution assay for the quantitative analysis of acetylpyrazine (I), 2-methyl-3-ethylpyrazine (II), 5-methyl-5*H*-cyclopenta[*b*]pyrazine (III), and 2-acetyl-1-pyrroline (IV) in the crusts of wheat and rye breads since as shown previously these compounds contribute significantly to the crustlike aroma note of the bread flavors (Folkes and Gramshaw, 1981; Schieberle and Grosch, 1983, 1984, 1985; Sizer et al., 1975). The present paper deals with the development of the assay for the flavor compounds I–IV and its application on different types of wheat and rye bread.

EXPERIMENTAL SECTION

Breads. The wheat breads were prepared with and without the addition of 10% (w/w) of a dried sponge as described earlier (Schieberle and Grosch, 1985). One type of rye bread was prepared in a one-stage sourdough process with the aid of citric acid and the other type by the three-stage sourdough process of Seibel et al. (1978). The rye grist bread was prepared from coarse whole rye flour as described earlier (Schieberle and Grosch, 1983).

Chemicals. 2-Acetylpyrrole, 2,3-pentanedione, ethylenediamine, 3-methyl-1,2-cyclopentanedione, and rhodium on activated alumina were from Fluka (Buchs, Switzerland). Pyrazinamide and methanol-d_1 (99.9% isotopic purity) were from Sigma (Munich, Germany). Ethylenediamine-d_4 (98% isotopic purity) and iodomethane-d_3 (98% isotopic purity) were from MSD Isotopes (IC Chemicals, Munich, Germany). Deuterium gas (99.7% isotopic purity) and ethylpyrazine were from Alfa (Ventron GmbH, Karlsruhe, Germany). Palladium on charcoal (10% Pd) was from Merck (Darmstadt, Germany), and neutral alumina was from Woelm (Eschwege, Germany). The solvents were purified as done by Schieberle and Grosch (1983). Silica gel 60 (Merck, Darmstadt, Germany) was treated with HCl and deactivated with 7% (w/w) water according to Esterbauer (1968).

Synthesis. Unlabeled acetylpyrazine (I), 2-methyl-3-ethylpyrazine (II), 5-methyl-5*H*-cyclopenta[*b*]pyrazine (III), and 2-acetyl-1-pyrroline (IV) were prepared and purified according to Roberts (1968), Flament and Stoll (1967), Flament et al. (1973), and Buttery et al. (1983), respectively. The MS and ^{1}H NMR data agreed with those published by the authors. Synthesis of the corresponding deuteriated compounds required the following modifications of these procedures:

Deuteriated Acetylpyrazine (I-*d*). Cyanopyrazine (2.1 g, 20 mmol), which was prepared from pyrazinamide (Roberts, 1968), was dissolved in 30 mL of diethyl ether. During 20 min this solution was added dropwise to a stirred and cooled Grignard solution that contained 1.5 g of magnesium and 8.5 g of methyl-d_3 iodide in 60 mL of diethyl ether. The reaction mixture was poured on 400 g of ice, and pyrazine I-*d* was isolated and recrystallized as described by Roberts (1968) for unlabeled pyrazine I.

Deuteriated 2-Methyl-3-ethylpyrazine (II-*d*). 2,3-Pentanedione (400 mg, 4 mmol) in 5 mL of diethyl ether was dropped at 0 °C into a solution of ethylenediamine-d_4 (240 mg, 4 mmol) in 5 mL of diethyl ether under stirring.

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