Chemical Synthesis of H^3- and 1-C^{14}-Labeled Polyunsaturated Fatty Acids

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Abstract

Total syntheses of polyunsaturated fatty acids labeled at the double bonds or the terminal methylene groups with tritium and the carboxyl group with C^{14} are discussed. The methods are applicable for all known unsaturated fatty acids with polyallylic structure and *all-cis* configuration.

Details for the synthesis of labeled γ-linolenic acid are given and methods of ozonolysis for determining the structure of unsaturated fatty acids are described.

Introduction

POLYENOIC FATTY ACIDS represent a large group of compounds that have been isolated from widely diverse sources. They are mainly present in the phosphatides of different cell structures (e.g., microsomes and mitochondria) and in the triglycerides of marine animals.

The chemical structures of these compounds, all very sensitive to oxygen, light and high temp, have been extensively and comprehensively studied (1). Polyunsaturated fatty acids are characterized by three distinct properties:

1) The olefinic bonds are arranged in an *allylic rhythm.*

2) The double bonds have *all-cis configuration.*

3) Most of the known acids belong to one of four groups which are defined by the number of C-atoms counted from the terminal methyl end to the next double bond: the palmitoleic (7C), oleic (9C), linoleic (6C), or linolenic (3C) acid types or families. These families reflect the limitations of biosynthetic pathways in higher animals. It is well established that all acids of the palmitoleic or oleic acid types can be synthesized by mammalian organisms starting from acetate; the polyunsaturated acids with 18, 20 and 22 C-atoms and 2 to 6 double bonds of the latter two types, however, use linoleic and linolenic acid as precursors. The latter two are essential and must be supplied from exogenous sources.

Little is known about the functions of polyunsaturated fatty acids; however, the mechanisms by which chain-elongation of essential fatty acids occurs have been elucidated in recent investigations (2–4) and the mechanism of desaturation is currently under intensive study. These studies were made possible by the development of methods for the total synthesis of C^{14} and H^3-labelled polyunsaturated fatty acids, the label being placed in an appropriate position of the molecule for a particular biochemical problem.

Polyunsaturated Fatty Acids H^3-Labeled at the Methyl End of the Molecule. The label in tritiated compounds must appear in a position that does not permit nonenzymatic exchange reactions under biological conditions. The labeling technique with H^3 is an attractive method for several reasons: it is economical, it permits the syntheses of labeled compounds with exceptionally high specific activities, and the H^3-acids can be used for clinical experiments in man. Two ways have been developed for tritium labeling:

1) Polyacetylenic acids can be obtained according to Osbond and Wickens (5,6) and catalytic half-hydrogenation in a tritium atmosphere produces acids *labeled at the double bonds* as shown in Figure 1.

2) H^3-labelled polyunsaturated fatty acids were synthesized (2) according to the schemes shown in Figure 2. Terminal H^3-linoleic-, γ-linolenic-, eicosa-11, 14-dienoic- and eicosa-8,11,14-trienoic acid have been synthesized by this method.

Polyunsaturated Fatty Acids Labeled at the Carboxyl End of the Molecule. A method used exclusively since 1952 for the synthesis of 1-C^{14}-linoleic-, linolenic-, and γ-linolenic acid has been developed by Howton et al. (8). A pure naturally occurring acid is completely brominated and then degraded to the 1-bromo-polybromalkane by the Hunsdiecker degradation. The double bonds are then regenerated with zinc and the resulting bromopolyene carboxylated with

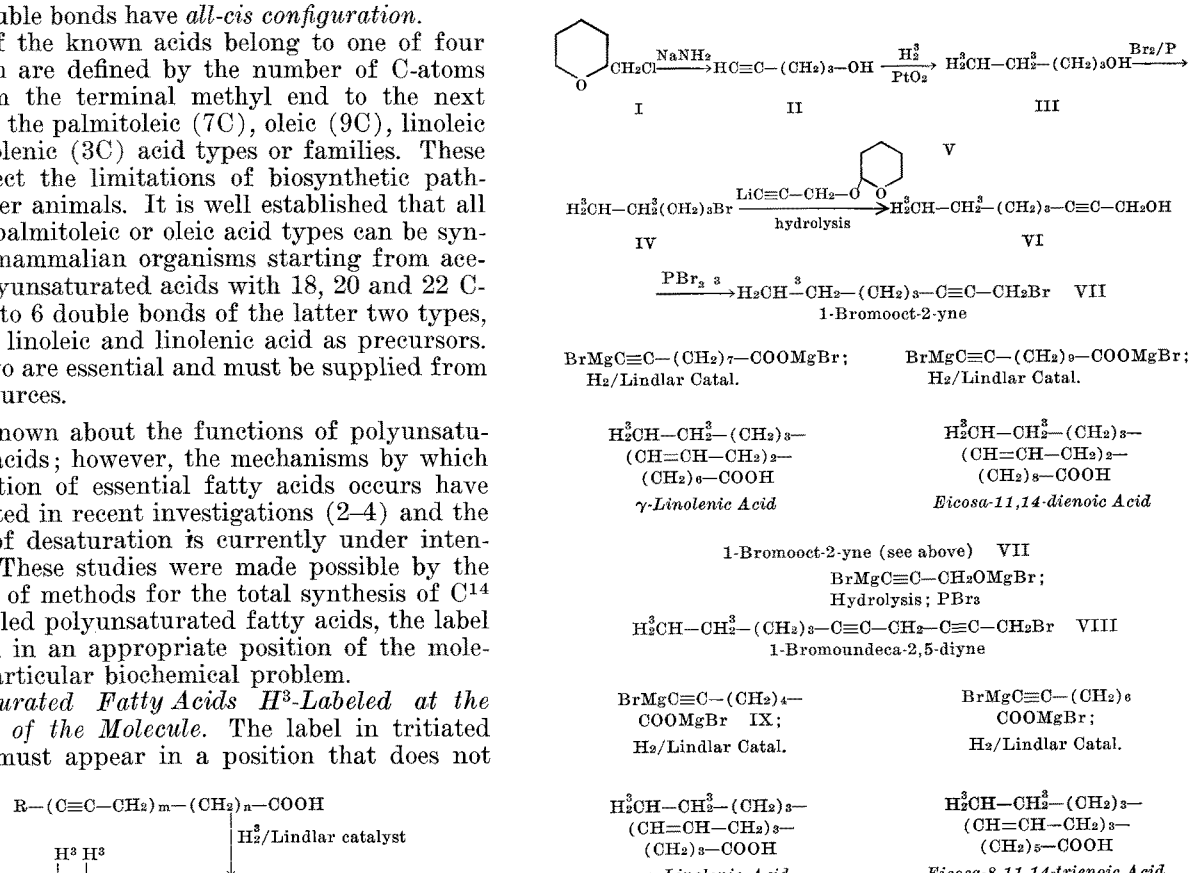

FIG. 1. Catalytic half-hydrogenation for the preparation of H^3-labeled all-*cis* polyenoic fatty acids.

FIG. 2. Total synthesis of all-*cis* C_{18} and C_{20}-dienoic and trienoic acids specifically labeled with tritium.

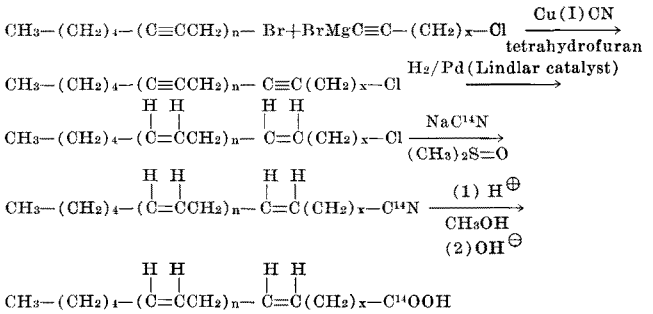

FIG. 3. Reaction scheme for the synthesis of 1-C^{14}-labeled polyenoic fatty acids.

$C^{14}O_2$ by a Grignard reaction. Beside the fact that pure natural acids are required, this method does not give products suitable for biochemical experiments. The yield does not exceed 25%; quantitative IR-spectroscopy indicates the presence of as much as 20% trans-isomers (10.4μ) and UV-spectroscopy indicates from 5 to 10% conjugated double bonds. Assuming a statistical distribution of the isomers, every fourth double bond represents an unnatural structure.

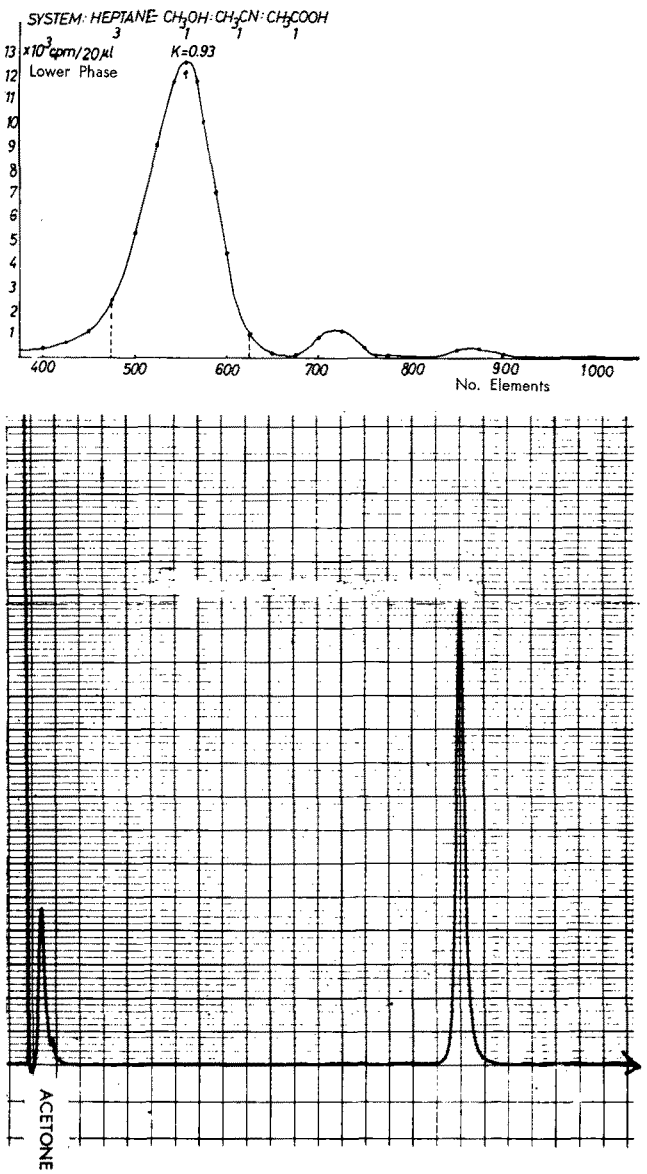

FIG. 4. (*a*) CCD-pattern of 1-C^{14}-γ-linolenic acid after 1100 transfers. Solvent system: heptane/acetonitrile/methanol/acetic acid, 3:1:1:1. (*b*) GLC analysis of methyl-1-C^{14}-γ-linolenate after CCD. Column temp 185C, EGS (15%), 200 cm column length, 60 ml argon/min, Barber Coleman Model 10.

We have developed a method for the total synthesis of 1-C^{14}-labeled polyunsaturated fatty acids without these undesirable features and with it we have been able to synthesize most of the known polyenoic acids (9,10). This labeling technique is the method of choice since it allows introduction of the marker, in high yield, in one of the last steps of a multistage synthesis. The sequence is outlined in Figure 3.

The principle of the synthesis is the condensation of a substituted propargylbromide (monoyne or polyyne) and an ω-chloro-alk-1-yne. The resulting chloropolyyne is partially hydrogenated with Lindlar-catalyst and the chloropolyene reacted almost quantitatively with $NaC^{14}N$ in dimethylsulfoxide. The nitrile is hydrolyzed to the methyl ester with HCl-methanol at room temperature and the free acid is obtained after mild alkaline hydrolysis.

The following C^{14}-carboxy-labeled polyunsaturated fatty acids have been synthesized: linoleic, γ-linolenic, eicosa-11, 14-dienoic, eicosa-8,11,14-trienoic, and arachidonic (eicosa-5,8,11,14-tetraenoic); linolenic, octadeca-6,9,12,15-tetraenoic, and eicosa-8,11,14,17-tetraenoic; iso-octadeca-6,9,12-trienoic; pentadeca,4,7,10-trienoic; hexadeca-7,10-dienoic and hexadeca-4,7,10-trienoic; octadeca-8,11,14-trienoic acid.

The nitrile synthesis with 1-chloro-heptadec-8-ene was very useful also for the synthesis of 1-C^{14}-oleic acid.

Gas liquid chromatographic (GLC) analysis proved to be the best method for analysis of the reaction products and intermediates. Other valuable analytical tools were IR-spectroscopy for the detection of trans and allenic structures (10.4μ, 5.1μ) and UV-spectroscopy of the isomerized and nonisomerized polyenoic acids. The preparations were also characterized by oxidative and reductive ozonolysis of the reaction products where subsequent GLC analysis proved the structures of the synthetic fatty acids. The details of our degradation procedures are given in the experimental part of this paper. Quantitative microhydrogenation according to Clausson-Kaas (11), melting points and mixed melting points of the hydrogenated acids served also for characterization of the preparations.

The purification of the intermediates as far as possible is mandatory in order to get chemically and radiochemically pure end products. Several chloropolyynes can be purified by low temp crystallization and the products freed from trace amounts of impurities by preparative GLC. Countercurrent distribution (CCD) has proved to be superior to these methods (12). Small amts of impurities (hydrogenated acids) are easily separated in the system heptane/methanol/acetic acid/acetonitrile, 3:1:1:1. Figures 4 and 5 are given as examples. They represent CCD-patterns of synthetic 1-C^{14}-γ-linolenic and arachidonic acids and the GLC tracings of the purified compounds.

Figure 6 shows the GLC analysis of dicarboxylic dimethyl esters obtained by oxidative ozonolysis and Figure 7 the IR spectrum of 1-C^{14}-arachidonic acid methyl ester.

The reaction sequences discussed here are suitable for the synthesis of any polyunsaturated fatty acid desired for biochemical studies. The availability of these compounds has enabled us to study the *in vitro* synthesis of polyunsaturated fatty acids and opens many other fields of lipid biochemistry. These pure substances must be used for reinvestigation of many problems hitherto based on relatively weak and less reliable data.

Experimental

Synthetic Procedures.

The synthesis of ^{3}H-γ-linolenic acid is described. The procedures are representative for the method.

3H-1-Bromo-octyne-2 (VII). Pentyn-1-ol-5 (II) was prepared from tetrahydrofurfurylchloride (I) according to Vogel (13). Catalytic hydrogenation of II in ethylacetate with tritium gas over platinum oxide yielded n-amylalcohol (III) with a specific activity of 2.3 μC/μmole. The alcohol was allowed to react with conc (48%) hydrobromic acid and yielded 90% n-amylbromide (IV), bp 78C/760 mm, $n_D^{20} = 1.4560$. The specific activity was 2.3 μC/μmole.

2-(Propyn-2-yloxy)-tetrahydropyran (V), which is the other component for synthesis of bromo-octyne, was prepared by reacting 2,3-dihydropyran and freshly distilled propargylalcohol. The strongly exothermic reaction was initiated with a drop of $POCl_3$. After 4 hr at room temp a few pellets of KOH were added for neutralization. Fractional distillation yielded 85% of V, bp 78C/25 mm, $n_D^{20} = 1.4560$.

The coupling reaction was carried out with lithium amide in 150 ml of liquid ammonia. A 250 ml three-necked flask was used with ground glass joints, a mechanical stirrer, and provisions to exclude atmospheric moisture. It was cooled in a dry ice-acetone bath of about −35C and 100 mg of ferric nitrate was added as catalyst to the ammonia. After addition of 0.82 g (0.12 g atoms) lithium, the mixture was stirred for one hour when 14.0 g (0.10 mole) of V were added dropwise over a period of 30 min. The temp of −35C and stirring were maintained for 4 hr after which 7.8 g (0.05 moles) of IV was added slowly within 20 min. Stirring was continued for an additional 5 hr and the ammonia was then allowed to evaporate overnight by removal of the cold bath. The excess of lithium amide was decomposed by air moisture during that time.

The reaction product was extracted with ether, the solvent evaporated and 10 ml of sulfuric acid (25%) was added with ice cooling. Methanol was added to obtain a one-phase system and the solution was stirred for 30 min at room temp followed by 60 min heating to reflux. After cooling, the aqueous phase was saturated with NaCl and the product (VI) was extracted with ether, washed with $NaHCO_3$, water and the solution dried over Na_2SO_4. Finally the octyn-2-ol-1 was fractionally distilled. Tritiated octynol was obtained at 91–92C/10 mm, $n_D^{20} = 1.4561$, in a yield of 5.0 g (38 mmoles, 78%).

The bromide was prepared by dissolving ^{3}H-octynol (VI) in 5 ml of dry ether which contained 0.2 ml of dry pyridine. Under rigorous exclusion of moisture, 4.0 g (15 mmoles) of PBr_3 was added over a period of 30 min while stirring and cooling to −20C. The reaction mixture was allowed to come to room temp and finally refluxed for 2 hr. After pouring the mixture on ice, the product was extracted with ether, washed twice with $NaHCO_3$, water, and dried over Na_2SO_4. The recovered oil was distilled and yielded 4.6 g (25 mmoles, 66%) of H^3-1-bromo-octyne-2 (VII), bp 90–92C/12 mm $n_D^{20} = 1.4862$. The specific activity was 2.3 μC/μmole and the compound proved to be pure according to gas chromatography.

^{3}H-1-Bromo-undecadiyne-2,5 (VIII). A 250 ml three-necked flask equipped with a mechanical stirrer, a condenser carrying a $CaCl_2$ tube and dropping funnel contained 2.4 g (0.10 g atom) magnesium and 25 ml of dry tetrahydrofuran. The Grignard reagent was prepared by adding dropwise 11 g (0.10 mole) ethyl-bromide in 25 ml of tetrahydrofuran. After beginning, the reaction was controlled by cooling the flask in a bath of −5C while further adding the ethyl bromide. Stirring was continued for 3 hr at room temp. A solution of 3.1 g (0.05 mole) propargylalcohol in 25 ml tetrahydrofuran was then added dropwise over a period of 30 min at 0C. After further stirring for 3 hr at room temp, 500 mg Cu^ICN was added. Ten minutes later, the coupling reaction was carried out by adding 4.5 g (0.025 mole) of VII. The mixture was refluxed under N_2 and then poured into 2N H_2SO_4 to decompose the magnesium compound. The product was recovered as usual and fractionally distilled. A yield of 3.3 g ^{3}H-undecadiynol (0.023 mole, 92%) was obtained, bp 92C/0.1 mm, $n_D^{24} = 1.4810$. It was converted into the bromide VII as described above for the conversion of octynol into VII. Distillation gave 3.3 g (15 mmoles, 74%) of VIII, bp 86C/0.6 mm, $n_D^{20} = 1.5155$.

6-Heptynoic acid (starting material for IX). A third triple bond was introduced with heptynoic acid. 1-Chloro-4-bromobutane was synthesized from tetrahydrofuran according to Newman and Wotiz (14). It was converted into 6-chlorohexyne-1 by reaction with sodium acetylide. The product had bp 144C/760 mm, $n_D^{20} = 1.4480$. 6-Cyano-hexyne-1 was obtained by reacting the chlorohexyne with NaCN in dimethylsulfoxide according to Smiley and Arnold (15). The nitrile was saponified by refluxing with a solution of 15% KOH in water-methanol (1:1) for 24 hr. Unsaponified material was extracted and heptynoic acid was recovered by acidifying the aqueous phase. The acid was purified by distillation, bp 88C/2.5 mm, $n_D^{20} = 1.4510$. The preparation was crystalline at 0C.

Methyl ^{3}H-6, 9,12-octadecatriynoate. The di-magnesium compound of 4.4 g (0.035 mole) 6-heptynoic

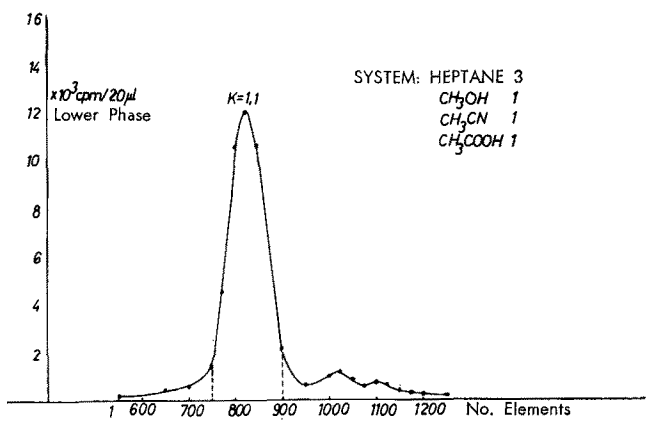

FIG. 5. (*a*) CCD-patter of 1-C^{14}-arachidonic acid after 1500 transfers. (*b*) GLC-analysis of methyl-1-C^{14}-arachidonate after CCD. Conditions as in Fig. 4.

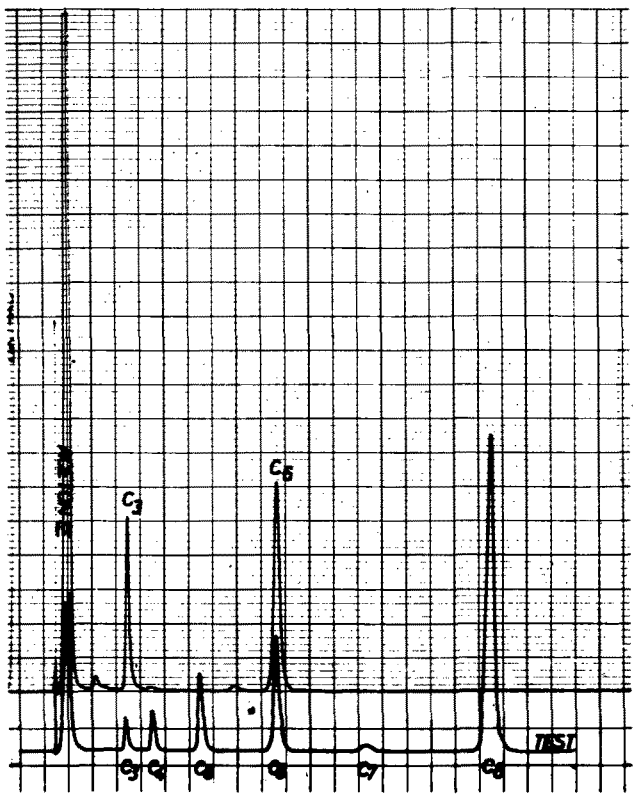

FIG. 6a. GLC analysis of dimethylesters of dicarboxylic acids after oxidative ozonolysis of (a) 1-C^{14}-γ-linolenic acid. Column temp 160C, EGS (15%), 200 cm column length, 60 ml argon/min, Barber Coleman Model 10.

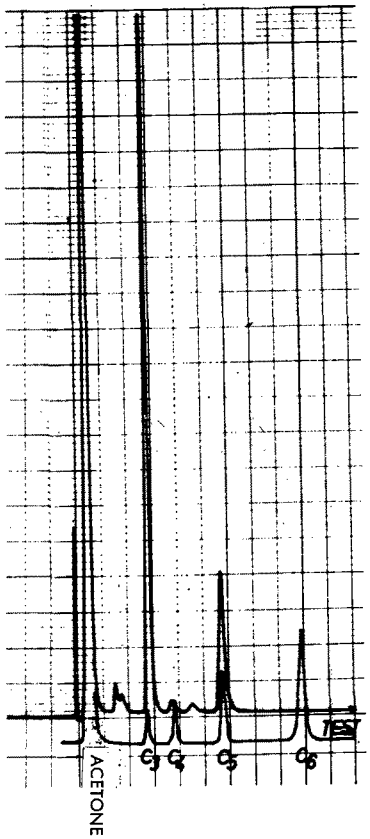

FIG. 6b. GLC analysis of dimethylesters of dicarboxylic acids after oxidative ozonolysis of 1-C^{14}-arachidonic acid. Same conditions as Fig. 6a.

acid (IX) was prepared in tetrahydrofuran under conditions as described for propargyl alcohol in the synthesis of VIII. Ten minutes after addition of 500 mg of $Cu^{I}CN$, 3.3 g (0.015 mole) ^{3}H-1-bromo-undecadiyne-2,5 (VIII) in 25 ml tetrahydrofuran was added. After refluxing for 8 hr under N_2, the magnesium compound was decomposed by pouring into 2N H_2SO_4 in ice-water. The acid was extracted with ether and re-extracted into 2N NH_4OH. It was again extracted into ether after acidification with 4N HCl under ice cooling. The ether solution was dried over Na_2SO_4, and the solvent then removed under vacuum. The residue was refluxed with 30 volumes of 5% methanolic HCl. The ester was recovered in the usual manner and distilled in an apparatus as described by Mohrhauer (16) at 10^{-4} mm, and a bath temp of 110–120C. The yield of triynoate (IX) was 3.3 g (0.012 mole, 80%), $n_D^{20} = 1.4680$, and GLC showed it to be pure. The specific activity was 2.3 μC/μmole.

all-cis Methyl ^{3}H-6,9,12-octadecatrienoate. Immediately after distillation, the ester was dissolved in 50 ml of distilled heptane. The partial hydrogenation was carried out with 2 g of Lindlar catalyst in presence of 0.05 ml of quinoline (synthetic, puriss grade). Within 40 min, 815 ml H_2 was consumed. Quinoline was extracted with 2N HCl and the heptane solution filtered and dried over Na_2SO_4. The solvent was evaporated under vacuum. The product was distilled like the triynoate at 10^{-4} mm and a bath temp of 110–115C. A yield of 3.2 g (0.012 mole) tritiated γ-linolenate was obtained. Gas-liquid chromatography showed it to be better than 95% pure and its specific activity was checked to be 2.3 μC/μmole.

Oxidative and Reductive Ozonolysis

The most reliable methods for elucidation of the structure of olefinic compounds are based on oxidative and reductive ozonolysis. The addition of ozone to olefinic bonds is not accompanied by isomerization. The combination of the two methods proved to be very effective in the determination of structures of polyenoic fatty acids (1).

The main purpose of oxidative ozonolysis (I) is the characterization of the so-called "carboxylic" end and the double bond arrangement. Reductive ozonolysis (II) unequivocally permits the determination of the paraffinic end of fatty acid chain by characterization of the dinitrophenylhydrazones of the resulting aldehydes. The schemes are shown in Figure 8.

The oxidative ozonolysis procedure given here is, except for some improvements, based on the method given by Klenk and Bongard (7), and that of reductive ozonolysis according to Klenk and Kremer (8).

I. *Oxidative Ozonolysis.* A fatty acid or a mixture of polyenoic acids (2–25 mg) is dissolved in 1 ml of methyl acetate, a small droplet of Sudan Red is added as indicator, and the solution cooled to −15 to −20C. Ozone is bubbled through the cooled mixture until the red color fades. Excess ozone is expelled with N_2. Acetic acid (1 ml) and 0.75 ml of 30% H_2O_2 are added and the reaction mixture incubated at 37C for 72 hr. Under these conditions about 40–50% of the malonic acid derived from intermediary parts of the polyenoic acid molecule is recovered. If the recovery of the carboxylic end only is desired, incubation of the mixture at 50–60C for about 12 hr is adequate for complete oxidation. A few mg of PtO_2, $Pd/BaSO_4$, or PdO are then added to decompose excess H_2O_2. Solvents are evaporated on a rotary still below 35C, and the residue is dissolved in water-methanol (1:1) and freed from catalyst by filtration. The monocarboxylic acids are separated from the dicarboxylic acids by extracting three times with petroleum ether (30–60C). The dicar-

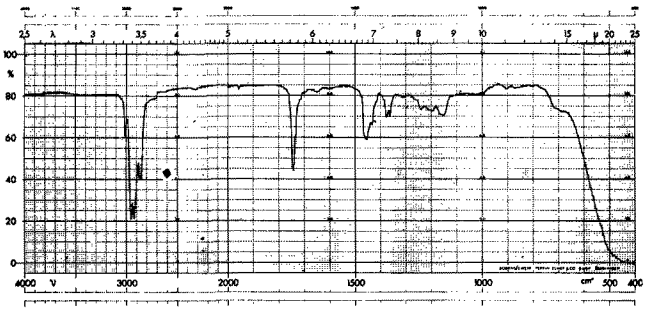

FIG. 7. IR spectrum of methyl-1-C^{14}-arachidonate, Perkin-Elmer 125.

boxylic acids are removed by evaporation of the aqueous methanol at 35C under vacuum. They are esterified in dry ether by treatment with diazomethane and examined quantitatively by GLC.

If determination of the yield is desired, the dicarboxylic acids are titrated with 0.01 N or 0.02 N methanolic KOH after the separation from monocarboxylic acids and complete evaporation of acetic acid. The absolute yield of each dicarboxylic acid can be calculated from the total number of equivalents of base used and the percentage obtained from the GLC analysis of the dimethyl esters. The latter are prepared in the following way: the K-salts of the dicarboxylic acids are taken to dryness on an evaporator, dry ether-HCl (5–10%) added, the free acids separated from KCl, and the solvent completely evaporated. The esters are then obtained with diazomethane in the usual manner.

II. *Reductive Ozonolysis of Unsaturated Fatty Acids.* Fatty acid (2–20 mg) is dissolved in 2 ml of methylene chloride and ozonized at −15C (a dry ice methanol cooling bath is used since acetone can give rise to contamination with acetone) with Sudan red as indicator. After completion of the reaction, excess ozone is expelled with N_2, 100–200 mg of solid triphenylphosphine is added and, after 1 hr at room temp, 10 ml of a 2,4-dinitrophenylhydrazone solution (6.0 gm in 30 ml conc HCl and 970 ml of 96% ethanol) is added. After 2 hr, 20 ml of H_2O is added and the 2,4-dinitrophenylhydrazones (DNPH) extracted five times with petroleum ether (60–80C) saturated with nitromethane. Two successive washings with a 10% Na_2CO_3 solution extract the DNPH-acids. The petroleum ether solution of the DNPH is dried over Na_2SO_4 and evaporated, the residue is dissolved in 1 ml of benzene, transferred to a 10 ml volumetric flask and made up to volume with petroleum ether (60–80C).

Partition Chromatography of DNPH. Twenty grams of silicic acid (Mallinckrodt 80–100 mesh), activated overnight at 110C, is thoroughly mixed with 16 ml of nitromethane. The solvent is added dropwise under rapid stirring with a glass rod and the mixture transferred to the chromatography tube (2 × 30 cm)

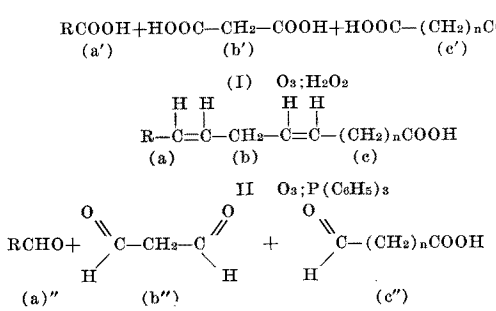

FIG. 8. Reaction scheme of oxidative and reductive ozonolysis (II).

TABLE I

DNPH	R_f	Factor (f)
C_2	0.23	1.28
C_3	0.38	1.31
C_4	0.53	1.30
C_5	0.74	1.30
C_6	1.00	1.50
C_7	1.37	1.55
C_8	1.80	1.60
C_9	2.4	1.65

as a slurry in petroleum ether (60–80C) which had been equilibrated with nitromethane. A column of 14 cm length is obtained by using nitrogen pressure. One to 3 ml of the DPNH solution is carefully layered on top of the column and chromatography begun under a slight N_2 pressure. The DNPH's separate as sharp yellow bands with the relative R_f values as are given in Table I for C_2 to C_9 aldehyde DNPH.

$$\text{relative } R_f = \frac{\text{distance, band center of component—top of column}}{\text{distance, band center of Hexanal DNPH—top of column}}$$

Fractions are concd and dissolved in benzene for quantification. The amt of DNPH is determined photometrically (at 365 mμ; d = 1 cm).

$$\text{mg DNPH} = \frac{f \cdot E \cdot V}{100}$$

The factors for different DPNH's are listed in Table I. E = extinction; f = factor; V = volume of hydrazone solution. R_f of acetone-DNPH is 0.64 and this band appears between butyraldehyde-DNPH and n-valeraldehyde-DNPH. Equation 2 permits the calculation of moles aldehyde per mole fatty acid.

$$\frac{\text{mole aldehyde}}{\text{mole fatty acid}} = \frac{\text{mg DNPH} \times \text{MW f.a.} \times 10}{\text{MW DNPH} \times \text{mg f.a.}}$$

With mixtures of polyenoic acids an average mol wt of 300 is assumed.

Although the different DNPH are characterized by their R_f, they can be further identified by reversed-phase TLC. TLC chromatoplates coated with Silica Gel (Stahl) are slowly and carefully soaked with a solution of vaseline (7%) in petroleum ether (60–90C). Plates which have only been air-dried but not activated at 110C are used preferentially for impregnation. A solvent composed of methanol-water, 7:3, proved satisfactory for separation by ascending TLC. The strongly yellow DNPH derivatives which had been spotted as 1–2 cm streaks can be located readily and compared with authentic compounds in the developed chromatograms.

REFERENCES

1. Klenk, E., Summary in Experientia *17*, 199–204 (1961).
2. Stoffel, W., Gemeins. Tag. Öster Dtsch. Ges. Phys. Chem. Wien, September 1962.
3. Stoffel, W., Z. Physiol. Chem. *333*, 71–81 (1963).
4. Stoffel, W., and K. L. Hch, Z. Physiol. Chem. *337*, 123–132 (1964) press.
5. Osbond, J. M., and J. C. Wickens, Chem. Ind. (London) 1288–1297 (1959).
6. Osbond, J. M., P. G. Philpott and J. C. Wickens, J. Chem. Soc. 2779–2787 (1961).
7. Stoffel, W., unpublished results.
8. Howton, D. R., R. H. Davis and J. C. Neuenzel, J. Am. Chem. Soc. *74*, 1109 (1952).
9. Stoffel, W., and E. Bierwirth, Angew. Chem. *74*, 905 (1962).
10. Stoffel, W., Liebigs Ann. Chem. *673*, 26–36 (1964).
11. Clausson-Kaas, N., and F. Limborg, Acta Chem. Scand. *1*, 884 (1947).
12. Ahrens, E. H., and L. C. Craig, J. Biol. Chem. *195*, 299–310 (1952).
13. Vogel, A. I., "Practical Organic Chemistry," p. 901–902, Longsman, Green and Co., New York, 1956.
14. Newman, M. S., and J. H. Wotiz, J. Am. Chem. Soc. *71*, 1292 (1949).
15. Smiley, R. A, and C. Arnold, J. Org. Chem. *25*, 157 (1960).
16. Mohrhauer, H., Dissertation, University of Cologne, 1960.
17. Klenk, E., and W. Bongard, Z. Physiol. Chem. *290*, 181–198 (1952).
18. Klenk, E., and G. Kremer, Z. Physiol. Chem. *320*, 111–125 (1960).