Chemical Synthesis of Tritium-labeled Linoleic Acid*

DEMETRIOS S. SGOUTAS AND FRED A. KUMMEROW

From The Burnsides Research Laboratory, University of Illinois, Urbana

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Tritium-labeled linoleic acid with a specific activity of 357 mc/mmole was obtained in 70% yield from totally synthesized octadeca-9,12-diynoic acid. A source of 1 curie of tritium gas was used to semihydrogenate the diacetylenic acid in the presence of Lindlar's catalyst. Chemical and radiochemical purity of the final product were assessed by chromatographic methods. Evidence from mild oxidation indicated that the tritium-labeled linoleic acid was prepared with a specificity of greater than 95% of the activity at the 9, 10, 12, and 13 positions.

The use of tritium-labeled compounds as tracers in biological studies is of growing interest. A simple process for the labeling of organic compounds by exposure to tritium gas has been described by Wilzbach (1957) and this technique has been applied for the production of tritium-labeled saturated fatty acid esters of a high specific activity (Nystrom *et al.*, 1959). Unsaturated fatty acid esters were found to preferentially hydrogenate, rather than substitute in the carbon chain, when exposed to tritium gas (Jones *et al.*, 1960; Dutton *et al.*, 1962). Interest in the preparation of labeled unsaturated fatty acids should therefore be centered on radiochemical syntheses (Tenny *et al.*, 1963).

In this paper the chemical synthesis of tritium-labeled linoleic acid by stereospecific partial reduction of the corresponding diacetylenic acid is described. Octadeca-9,12-diynoic acid was prepared by condensing 1-bromo-oct-2-yne with the di-Grignard derivative of 9-decynoic acid in anhydrous tetrahydrofuran with cuprous cyanide as catalyst. This condensation has been investigated by Osbond and co-workers (Osbond and Wickens, 1959; Osbond *et al.*, 1961). The starting materials (1-bromo-2-octyne and 9-decynoic acid) were obtained by modifying known procedures through the route presented in Figure 1. Black and Weedon (1953) have reported the degradation of the ethyl ester of 10-undecynoic acid. Their procedure was modified for the degradation of the methyl ester of 10-undecynoic acid in this study. The resulting 9-decynoic acid was purified by conversion to its methyl ester and fractional distillation of the latter. Azeotropic methylation was particularly suitable for obtaining pure methyl esters of ω-acetylenic acids in high yields. When methanol saturated with hydrogen chloride was used as the methylating agent side reactions yielded by-products which contained chlorine, indicating that addition of hydrogen chloride to the triple bond of the ω-acetylenic acid had occurred.

For the partial hydrogenation of the diacetylenic acid, Lindlar's catalyst was used; almost pure octadeca-9,12-dienoic acid with a *cis* configuration was obtained. A tritium source which contained approximately 1 c was employed to introduce 357 mc of activity per mmole of linoleic acid. The chemical yield (*ca.* 70%) of the reaction under these experimental conditions was not so satisfactory as when only hydrogen was used (*ca.* 82%). However, under the former conditions the pressure drop inside the closed system in which the reaction with the radioisotope took place may have been responsible for the lower yield. Also, evidence was obtained by gas-phase chromatography that a relatively larger amount of oleic and stearic acids were formed in the presence of tritium. Radiation damage and radiation decomposition products were absent, and treatment with ethanol was effective in removing the labile tritium. Convenient methods such as crystallization at low temperature and fractional distillation of the methyl ester were quite satisfactory in yielding a chemically and radiochemically pure product.

Since experience in radiochemistry has shown that the employment of several criteria of purity is desirable, if not mandatory, use of gas-phase chromatography, thin-layer chromatography (both reverse-partition and adsorption), and column chromatography (adsorption) were offered to support the conclusion that the linoleic acid was radiochemically pure. Furthermore, bromination-debromination demonstrated not only the radiochemical purity of the product but also the stability of the label to chemical manipulation. Oxidative degradation of the purified linoleic acid indicated that greater than 95% of the tritium radioactivity was at the 9, 10, 12, and 13 positions, and chromatography on silica impregnated with silver nitrate showed that only traces of radioactivity were associated with the *trans* isomer.

Thus synthesis of tritium-labeled linoleic acid with a high specific activity and with the label at specific positions was realized. Use of this acid as a tracer in biological studies may become important in connection with studies on the essential fatty acids in the controversial area of the relationship between polyunsaturated fatty acids, blood cholesterol, and atherosclerosis.

EXPERIMENTAL

Ultraviolet absorption determinations were carried out in spectroscopically pure ethanol on a Cary Model 11 M recording spectrometer. The infrared measurements were carried out on a Beckman Model IR 7 recording spectrometer on Nujol mulls. All indices of refraction were determined with a Zeiss refractometer. An Aerograph Model A-90-C (Wilkens Instruments and Research, Inc.) equipped with a Daystrom-Weston recorder, a thermal conductivity unit, with filaments operating at a current of 200 ma and at temperature of 190°, was employed for gas-phase chromatography. The column, 0.5 in. inner diameter and 5 ft in length, was packed with a 20% diethylene glycol succinate polymer on 60–80 mesh firebrick support. The carrier gas was helium and had an effluent flow rate of 35 ml/minute. Radioactivity was determined with an automatic Packard Tri-Carb liquid scintillation spectrometer Model 314 E (Packards Instrument Co., Inc). Sample vials of low-potassium

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glass were used. The photomultiplier high voltage was set at Tap 9.1. Discriminator settings were at 20–1000; gain 7%. An efficiency for tritium of around 34% was achieved.

10-Undecynoic Acid.—This compound was prepared from commercial 10-undecenoic acid by bromination-debromination according to Vogel (1956), the only difference being that the intermediate dibromoderivative was purified (mp 38–38.5° uncorr) by crystallization from light petroleum ether. This purification eliminated foaming during the debromination.

Methyl-10-Undecynoate.—Methylation of 10-undecenoic acid was accomplished by the azeotropic method. Twenty-five g of 10-undecenoic acid in 130 ml chloroform and 100 ml anhydrous methanol containing 1.0 ml of concentrated sulfuric acid was refluxed for 16 hours in a Soxhlet apparatus containing anhydrous magnesium sulfate in the thimble. Isolation of the ester was carried out in the usual way. Yield 91.2%; bp 100–101°/3mm; n^{20}_D = 1.4460. Gas-phase chromatography showed one peak.

9-Decynoic Acid.—Methyl-10-undecynoate was degraded according to Wieland's (Weiland *et al.*, 1926) modification of the method of Barbier and Locquin (1913) to yield 9-decynoic acid. Black and Weedon (1953) have reported the degradation of the ethyl ester of 10-undecynoic acid and their procedure was modified for the degradation of the methyl ester. 1,1-Diphenyl-10-undecyn-1-ol was prepared by the addition of a solution of 27 g of methyl-10-undecynoic acid in 70 ml of dry ether to the Grignard reagent of bromobenzene (10 g of magnesium turnings, 65 g of bromobenzene in 400 ml ether) with external cooling to −5° during the course of 2 hours. After the addition had been completed, the reaction mass was heated under reflux for 2 hours on a water bath at 50°. Decomposition of the magnesium complex and recovery of the carbinol was performed as reported by Black and Weedon (1953). Distillation under vacuum gave 32.2 g (72%) of 1,1-diphenyl-10-undecyn-1-ol; bp 135–140°/0.2 mm; n^{20}_D = 1.5520. This was dehydrated as recommended by Crombie and Jacklin (1957), the only difference being that iodine was added as catalyst to the reaction. 1,1-Diphenylundec-1-en-10-yne was oxidized according to the procedure of Black and Weedon (1953) to yield (62.3%) 9-undecynoic acid; bp 95–98°/0.5 mm; n^{20}_D = 1.4516; mp 19–20°; reported (Black and Weedon, 1953) 22°. A small sample was methylated by the azeotropic method and gas-phase chromatography showed the sample to be about 90% pure with two minor contaminants. Attempted recrystallizations from several solvents as well as fractional distillation of the acid failed to give a product of higher purity. Consequently purification through the methyl ester was attempted. A solution of 8.0 g of 9-decynoic acid in 40 ml chloroform and 35 ml methanol containing 0.5 ml of concentrated sulfuric acid was refluxed for 16 hours in a Soxhlet apparatus containing anhydrous magnesium sulfate in the thimble. The crude methyl ester isolated in the usual manner was distilled through a 25-cm Vigreux column and the fraction distilling at 91–92°/3 mm was collected. Yield 8.1 g (94%). According to gas-phase chromatography the fraction was shown to be more than 98% pure. Conversion of the methyl ester to the free acid was accomplished by saponification. Fifteen ml of 20% aqueous sodium hydroxide solution was poured into a 100-ml flask and warmed, and 8.1 g of methyl 9-decynoate was added dropwise to the warm alkali solution. The mixture was slowly refluxed by heating for 2 hours, and when the ester layer disappeared the product was cooled and carefully acidified with

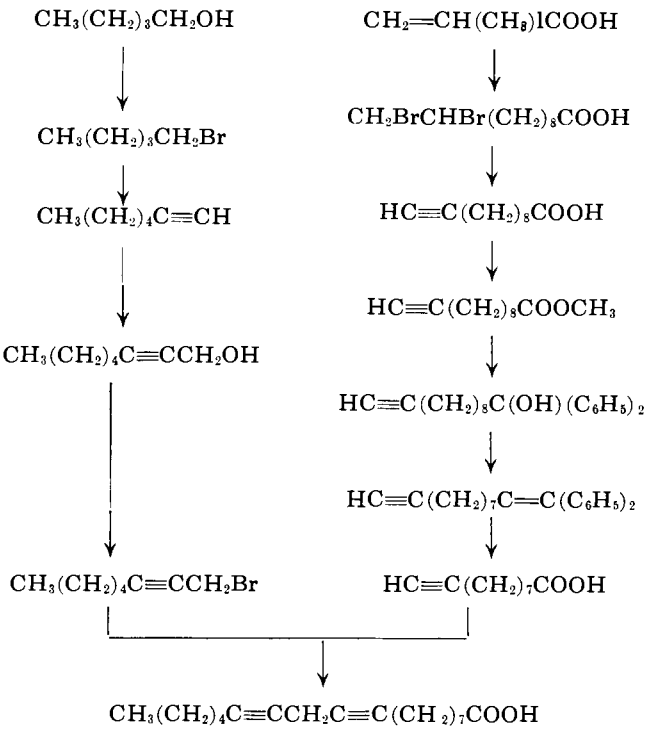

FIG. 1.—Sequence in the reactions involved in the total synthesis of octadeca-9,12-diynoic acid.

concentrated hydrochloric acid. After cooling to room temperature the acid was extracted with ether, the ethereal extract was dried over anhydrous sodium sulfate, and the solvent was removed. The crude acid was distilled, bp 100–101°/3 mm; yield 7.0 g (92%); mp 21–22°.

1-Heptyne.—The method given by Campbell and Campbell (1950) was modified for the synthesis of this compound. Yield 65%; bp 98–99°.

2-Octyne-1-ol.—This compound was synthesized by a modification of the procedure of Taylor and Strong (1950). A solution of ethyl magnesium bromide was prepared from 8 g of magnesium turnings and 50 g of ethyl bromide in 200 ml of ethyl ether. This solution was cooled to −5° and 27 g of 1-heptyne in 30 ml ethyl ether was added with vigorous stirring over a period of 1 hour. The solution of heptynyl magnesium bromide was cooled to −10° and gaseous formaldehyde produced by depolymerization of 30 g of paraformaldehyde was introduced over a period of 2 hours. After the addition was completed the reaction mixture was heated under reflux for 30 more minutes and decomposed with ice and dilute sulfuric acid at −5°. Distillation of the isolated product gave 2-octyne-1-ol in 51% yield; bp 80–81°/5 mm.

1-Bromo-2-octyne.—Bromination of 2-octyne-1-ol was performed as reported by Taylor and Strong (1950).

Octadeca-9,12-diynoic Acid.—The procedure of Osbond *et al.* (1961) was used for the condensation of 1-bromo-2-octyne and 9-decynoic acid in anhydrous (distilled over $LiAlH_4$) tetrahydrofuran and cuprous cyanide as catalyst. The methyl ester of octadeca-9,12-diynoic acid was prepared with superdry methanolic HCl under a stream of nitrogen by refluxing the reaction mixture at 80° for 2 hours. Gas-phase chromatography showed one main peak with a relative retention time of 3.6 (methyl linoleate = 1.0).

Tritium Linoleic Acid.—Octadeca-9,12-diynoic acid (0.5 g) and 0.1 g of Lindlar's catalyst were placed in a flask with a break-seal-joint adaption. The flask was flushed with helium and cooled with liquid nitrogen, a

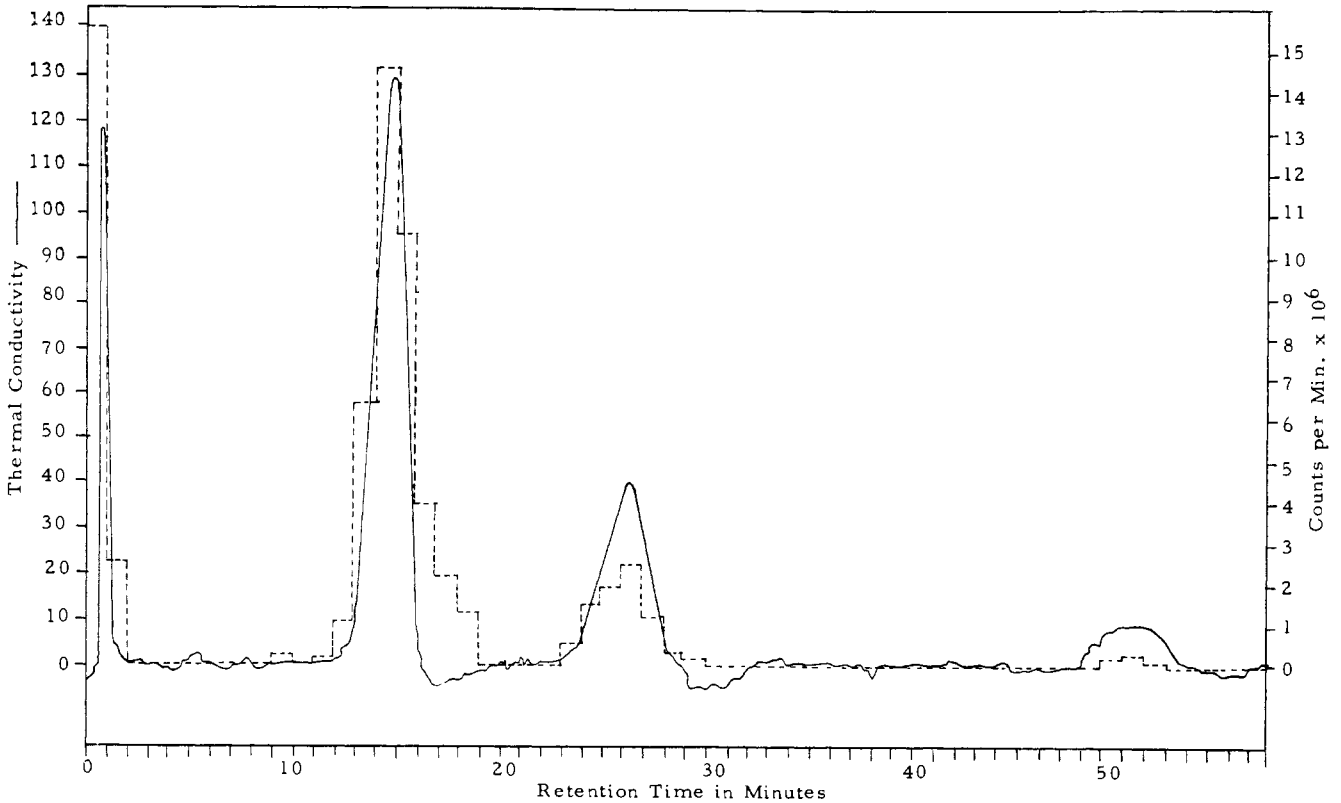

FIG. 2.—Gas chromatogram of crude tritium-labeled methyl linoleate.

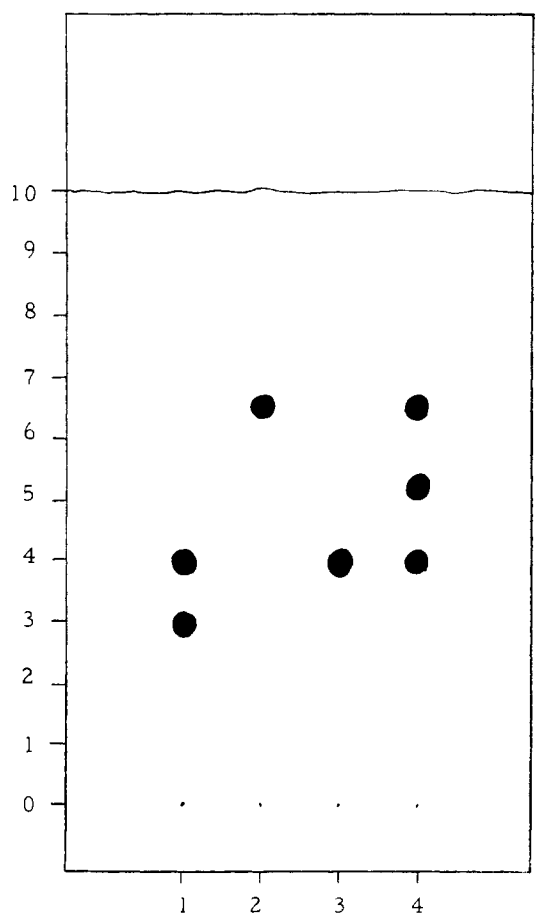

FIG. 3.—Reversed-phase partition chromatography on a siliconized chromoplate. Solvent, glacial acetic acid–water, 80:20, v/v. (1) Methyl oleate and methyl linoleate, (2) methyl-octadeca-9,12-diynoate, (3) methyl linoleate, (4) mixture from the tritiation of octadeca-9,12-diynoic acid after methylation.

vacuum was drawn (0.001 mm Hg), and 25 ml of redistilled *n*-hexane containing 0.4 ml of a 5% solution of quinoline in *n*-hexane was transferred into the flask through the high-vacuum line. The constricted neck of the flask was closed off while the flask was chilled with liquid nitrogen. The Toepler pump was connected and 1 cc of tritium gas (1 c) was forced in through the break-joint adaption. Hydrogen gas (300 cc) was then added by the same means and the flask was completely sealed. The solution was stirred over the course of 6 hours, at room temperature, whereupon excess amounts of hydrogen and tritium were pumped off. The solution was freed of catalyst by filtration through a sintered filter, and the solvent was evaporated. An aqueous methanolic solution of hydrochloric acid was added to remove quinoline and this solution was extracted with distilled *n*-hexane. The extract was washed with a little distilled water and dried over sodium sulfate. An aliquot of 1 ml (from a total of 50 ml solution) was methylated with 5% methanolic hydrogen chloride at 60° for 2 hours; gas-phase chromatography showed one main peak with two small subsidiary contaminants. The main peak possessed the same retention time as the reference methyl linoleate and the highest retention time of a contaminant was the same as that of methyl-octadeca-9,12-diynoate. The other peak presumably was due to an intermediate product of the reduction, i.e., 9,12-octadecenynoic acid. Upon complete hydrogenation in methanol over platinum oxide, the three peaks disappeared and only a peak corresponding to methyl stearate appeared.

Simultaneously with the recording of thermal conductivity, radioactivity was determined by directly trapping methyl esters from the effluent gas stream in vials containing 15 ml of scintillation solution (3 g PPO[1] and 50 mg POPOP/liter of redistilled toluene)

[1] Abbreviations used in this work: PPO, 2,5-diphenyloxazole; POPOP, 1,4-2-(5-phenyloxazolyl)-benzene.

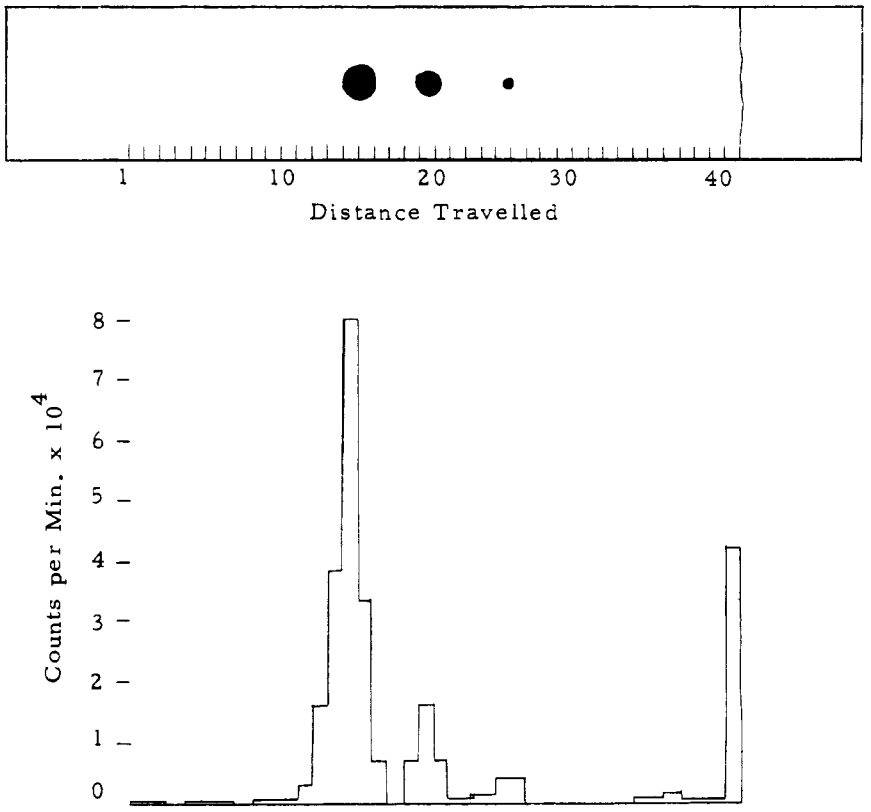

FIG. 4.—Record of radioactivity obtained from a thin-layer plate by the technique described. Chromatogram as in Fig. 3; sample 4 is attached to top.

for specified periods, e.g., 30 seconds, and by subsequent assay in the scintillation spectrometer. The major radioactive product on gas chromatography came at the position of methyl-octadeca-9,12-dienoate. A comparatively large amount of radioactivity was associated with the solvent front and the intermediate product. The results of gas chromatography and subsequent liquid scintillation counting of the crude methyl linoleate are given in Figure 2.

In addition, thin-layer chromatography was employed as another method of checking the chemical and radiochemical purity of the methyl octadecadienoate-^{3}H. Glass plates were coated with silica-gel G and impregnated with silicone oil according to the method of Malins and Mangold (1960) (Figure 3). Radioactivity was assayed by a modification of the method of Snyder and Stephen (1962). The material was applied on the thin-layer plate in two separate spots. The chromatogram was developed until the solvent front had migrated approximately 20 cm and the plate was then dried. One half of the chromatogram was covered with a glass plate and the other half was sprayed revealing the position of components derived from one of the original spots. The second half of the plate, containing the chromatogram from the second spot, was divided into sections parallel to the solvent front. Each section was scraped from the glass plate into a scintillation vial and the radioactivity was determined. A comparison of the distribution of radioactivity and of the chemical components of the original mixture was thus obtained (Figure 4).

Purification of Tritium-labeled Linoleic Acid.—The small amount of rapidly eluted radioactive material which coincided with the solvent peak was thought to be either residual tritium-gas or solvent molecules containing labile tritium. Distillation of 400 ml of hexane from the reaction product removed the low boiling point radiochemical impurities, and distillation of 600 ml of anhydrous ethanol in 50-ml batches freed the preparation from labile tritium which rapidly exchanged with ethanol. Both distillations were conducted in an atmosphere of nitrogen. The residue was dissolved in 20 ml of *n*-hexane and stored at −20° overnight; 0.05 g of solid material was precipitated, removed by filtration, and upon recrystallization from *n*-hexane melted at 42–43°. After a small aliquot was mixed with an approximately equal amount of octadeca-9,12-diynoic acid (mp 42–43°) the mixture melted at 42–42.5°. The solution was kept at −25° for another 24 hours and 0.025 g of a yellow liquid was removed. The solution was brought to −80° for 5 minutes and then kept at −60° for 20 hours. White crystals precipitated and the mother liquid was removed by suction. Addition of *n*-hexane and recrystallization at −60° gave 356 mg of linoleic acid mp −8°, reported −5.2 to −5° (Matthews *et al.*, 1941). One-tenth g of the linoleic acid was methylated with 5% methanolic hydrogen chloride, and gas-phase chromatography showed the methyl ester to be of a calculated 92% purity. By means of the "mixed chromatography" technique in which inactive known esters are added to unknown mixtures, the position of the major radiochemical component (*ca.* 94.5%) of the tritium-labeled methyl linoleate was shown to coincide in elution (in both gas-phase and thin-layer chromatography) with that of inactive methyl linoleate, and a small amount of radioactivity coincided with the position (peak or spot) of inactive acetylenic intermediate.

Further purification was effected by preparation of the methyl ester of the tritium-labeled linoleic acid (5% methanolic hydrogen chloride at 20° overnight) and fractional distillation after dilution of the radioactive material (0.2 g) with inactive pure methyl linoleate (2.0 g), prepared by partial hydrogenation of octadeca-9,12-diynoic acid (Osbond *et al.*, 1961);

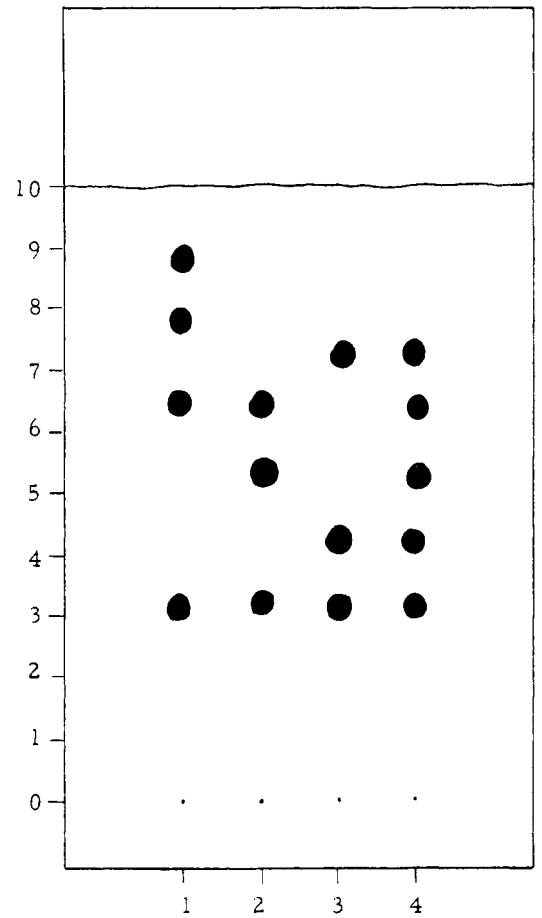

FIG. 5.—Thin-layer chromatography of methyl esters on a silica–silver nitrate adsorbent. Solvent, Skellysolve B–ether, 85:15, v/v. (1) Mixture of methyl linoleate, linolelaidate, oleate, and elaidate; (2) product of the linoleic acid elaidinization after methylation; (3) mixture from the tritiation of octadeca-9,12-diynoic acid after methylation; (4) mixture of (2) and (3).

bp 140–142°/0.1 mm; iodine value 171; n^{20}_D = 1.4600. The ultraviolet absorption at 233 mμ was nil and the infrared spectrum indicated less than 1% *trans* isomer; this estimate was based on absorption at 10.3 μ. Gas-phase chromatography showed one peak with no contaminants. Radioassay by means of the "mixed chromatography" technique showed it to be (*ca.* 99%) pure both on gas-phase and thin-layer chromatography. The methyl linoleate thus purified (after dilution and distillation) was found to contain 35.7 mc/mmole.

Bromination-Debromination.—Ten mg of the radioactive methyl linoleate (specific activity 35.7 mc/mmole) was mixed with 5.3 g of inactive methyl linoleate to form a new dilution with activity of 66.57 μc/mmole (calcd 67.2). This was saponified and the recovered linoleic acid after distillation contained 66.10 μc/mmole. Bromination was performed in *n*-hexane, and the tetrabromostearic acid was recrystallized twice from 80% acetone (mp 114–115°) and its radioactivity was determined by liquid scintillation spectrometry. It was found to be 65.90 μc/mmole. A quantity of 1 g of the tetrabromide was debrominated to obtain linoleic acid according to the method of Matthews *et al.* (1941). The linoleic acid thus isolated was distilled and its specific radioactivity was determined by scintillation counting. It was found to be 65.3 μc/mmole.

Silica–Silver Nitrate Chromatography.—In order to study the configuration of the double bonds in the octadeca-9,12-dienoate-^{3}H, silica impregnated with silver nitrate was used. A portion, 0.003 g and 360 μc, of the pure product was added to 0.3 g of a methyl linoleate–linolelaidate mixture (7:3, v/v), 50 μg of this solution was chromatographed on plates coated with silica impregnated with silver nitrate (Barrett *et al.*, 1962) (Fig. 5), and radioassay in the manner described previously was performed. One main radioactive peak appeared at the position of *cis-cis*-octadeca-9,12-dienoate and traces (less than 1%) at the position of *trans-trans* methyl ester.

Alternatively, column chromatography on silica gel impregnated with silver nitrate was applied by using similar conditions with those that enabled De Vries (1963) to separate methyl elaidate from methyl oleate. One radioactive peak which coincided in position with that of *cis-cis*-octadeca-9,12-dienoate appeared and traces of radioactivity in the position of all *trans*-octadeca-9,12-dienoate indicated the presence of the *trans* form in less than 1%.

Degradation of Tritium-labeled Linoleic Acid.—Oxidative cleavage of the double bonds in the tritiated methyl linoleate was accomplished by the method of von Rudloff (1956) after addition of inactive ester to tritiated ester to give a final activity of 1.36 × 10^6 dpm/mmole. This method involved saponification of the ester, oxidation of the acids as soaps at room temperature with periodate-permanganate solution, and subsequent extraction of all ether-soluble acids. A small sample of the crude oxidation product was methylated with diazomethane and was analyzed by gas-phase chromatography. Two major peaks appeared which were identified by standards as methyl caproate and dimethyl azelate. It was found that methyl caproate had an activity of 24,860 dpm/mmole corresponding to an average of 1.8% of the radioactivity in the linoleic acid, while dimethyl azelate possessed an activity of 41,800 dpm/mmole corresponding to an average of 3.1% of the radioactivity in the linoleic acid. A large amount of radioactivity was associated with the solvent front in all the above chromatographic runs. The crude oxidation product was extracted with light petroleum ether and a small portion of the material that was removed was methylated and subjected to gas-phase chromatography. It was found to be *ca.* 90% pure methyl caproate. Another small portion of the material which was extracted with petroleum ether was passed through a column of silicic acid (von Rudloff, 1956) and alternate 5-ml eluate fractions were titrated and radioassayed with the scintillation spectrometer. The mass and radioactive peaks coincided and the specific activity determined for the caproic acid was found to be 29,500 dpm/mmole. The residue from the light petroleum ether extractions was recrystallized from ether and showed a mp of 105–106°. No depression was observed on admixture of an equal amount of this residue with an authentic specimen of azelaic acid. The azelaic acid thus isolated was recrystallized repeatedly from ether and toluene to constant specific radioactivity and it was found to contain 65,200 dpm/mmole (4.8% of the radioactivity in the original linoleic acid). Isotope dilution assay gave similar results. To aid in the clarification of the above observed discrepancy anhydrous ethanol was distilled over the sodium salt of azelaic acid until no radioactivity could be detected in the distillate. The specific activity of the azelaic acid was determined and it was found to be 43,500 dpm/mmole (2.9% of the radioactivity in the original linoleic acid, which was consistent with the gas-phase chromatographic data).

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Purification and Characterization of the Lipid A Component of the Lipopolysaccharides from *Escherichia coli**

ALICE J. BURTON† AND H. E. CARTER

From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana

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A crude lipopolysaccharide fraction has been isolated from *Escherichia coli*, strain $O_{111}B_4$, by cold aqueous phenol extraction. Purification of this material gave a lipopolysaccharide preparation (F-III) of high molecular weight in 4–5% yield (based on the dry weight of the cells). In addition, by extending the purification procedures commonly used, another 10–15% yield of lipopolysaccharide was recovered. The lipid content of this second preparation (F-VI) has not been previously observed. Mild aqueous acid degradation of the bacterial lipopolysaccharides released the Lipid A component, which was then purified by acetone fractionation and silicic acid chromatography. A major portion of the lipid was consistently obtained as a well-defined peak from silicic acid columns. The purified lipid derived from F-III and designated as A-III melts at 197–200°, has a molecular weight of 1700, gives the triphenyltetrazolium test for a reducing end group, and shows limited solubility in organic solvents. It is devoid of glycerol, necrosamine, and peptides. The analytical data are consistent with a structure containing two glucosamine molecules, one phosphate, three to four acetyl groups, and five long-chain fatty acids (of which about half are hydroxy acids). Calcium and magnesium are present. On the basis of preliminary results from sodium borohydride reduction and alkaline hydrolysis of the lipid, a structure has been tentatively proposed for Lipid A in which the basic unit consists of two glycosidically linked fully acylated glucosamine molecules. Lipid prepared from F-VI is similar in chemical composition and solubility properties to A-III, but differs in that its molecular weight is approximately twice that of A-III.

From the cells of Gram-negative bacteria it is possible to extract lipopolysaccharide complexes which have marked biological activity. Their antigenic and endotoxic characters are especially important, but these complexes are also pyrogenic, often promote resistance to infection, and will inactivate phages which infect the original bacteria. These bacterial complexes contain a major lipopolysaccharide component associated with protein or peptide and biologically inert lipid in amounts varying with the solvent used in extraction. The chemistry and biological effects of these bacterial complexes are under investigation in a number of laboratories (Westphal, 1960; Osborn *et al.*, 1962; Ribi *et al.*, 1962; Nowotny, 1963).

Westphal and Lüderitz (1954) have shown that mild acid degradation of lipopolysaccharide yields a lipid fraction (designated as Lipid A). Our particular interest in this problem concerns the preparation of Lipid A in a pure form and the characterization of its structure. For this study lipopolysaccharides have been prepared from *Escherichia coli* by cold aqueous phenol extraction. The lipid components of the lipopolysaccharides have been prepared and their composition examined. In this report, we describe the preparation and properties

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† Present address: Division of Biology, California Institute of Technology, Pasadena, California.