Characterization of a Long-Chain ^{14}C -Labelled Alcohol Resulting from a Plant Enzyme-Catalysed Reaction with *S*-Adenosyl[methyl- ^{14}C]Methionine

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(Received 8 November 1971)

A particulate enzyme from *Phaseolus aureus* seedlings catalyses the incorporation of radioactivity from *S*-adenosyl[Me- ^{14}C]methionine into several lipid acceptors present in the enzyme preparation. The structure of one of the radioactive lipids has been elucidated by using mass, n.m.r. and i.r. spectral data. This compound has a molecular formula $\text{C}_{28}\text{H}_{57}\text{OH}$, is a saturated primary alcohol and has a single branch of one methyl group in the interior of the hydrocarbon chain. The results of a Kuhn–Roth degradation demonstrate that the branch methyl group is the one derived from *S*-adenosylmethionine.

Enzymes have been isolated from bacteria (Akamatsu & Law, 1968, 1970; Chung & Goldfine, 1965; Law, 1967; Polacheck *et al.*, 1966; Thomas & Law, 1966) that catalyse the alkylation of fatty acid moieties present in molecules such as phosphatidylethanolamine, phosphatidylglycerol, other phospholipids, and of alkyl ethers in plasmalogens. These reactions utilize *S*-adenosylmethionine as a methyl donor and involve a C_1 addition to a double bond. The C_1 piece can appear in the product as one member of a cyclopropyl ring (Chung & Goldfine, 1965; Law, 1967; Thomas & Law, 1966) or as a methyl group attached to a methylene chain (Akamatsu & Law, 1970). Methyl transfers with *S*-adenosylmethionine as a methyl donor are commonly catalysed by plant enzymes (Bonner & Varner, 1965), but the products resulting from the enzymes that have been isolated have usually been *O*-methylated molecules such as a sugar (Kauss & Hassid, 1967), or flavonoids, lignins and other methoxylated aromatic constituents (Bonner & Varner, 1965). However, enzymes from plants catalyse the alkylation of sterol side chains to produce C_{28} and C_{29} sterols (Alexander & Schwenk, 1958). These reactions are presumed to proceed with mechanisms similar to those determined for the bacterial enzymes. Although branched-chain fatty acids and hydrocarbons (Kaneda, 1968) can be found in higher plants, no enzymes have been isolated that will catalyse the transfer of methyl groups to compounds of this type. While investigating the synthesis of plant cell-wall polysaccharides, we observed that the particulate enzyme preparation employed would catalyse what appears to be transmethylation reactions involving *S*-adenosylmethionine and several lipid acceptors present in the preparation. This report describes the characterization of a methylated compound that resulted from this reaction.

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Methods

Enzyme preparation

The particulate enzyme was prepared essentially as described previously (Villemez *et al.*, 1966). The cotyledons were removed from *Phaseolus aureus* seedlings, which had been germinated for 4 days at 20°C in the dark. Shoots (25g) were combined with an equal weight of cold buffer [0.05M-potassium phosphate (pH 7.3)–1% bovine serum albumin and ground for several minutes in a cold mortar with 1g of sand. The homogenate was filtered through several layers of cheesecloth and centrifuged at 1000g for 15 min. The particulate enzyme was isolated from the supernatant solution by sedimentation at 34000g for 45 min. The pellet was resuspended in 1ml of buffer [0.05M-potassium phosphate (pH 6.8)–1% bovine serum albumin–0.4M-sucrose] and used as the enzyme course.

Reaction conditions

A portion (7ml) of enzyme preparation was combined with 7ml of 0.1M-tris-HCl buffer (pH 8.4) and 70 μl of 0.1M- CoCl_2 . The mixture was preincubated at 30°C for 3 min and 1ml of an aqueous solution of *S*-adenosyl[Me- ^{14}C]methionine (1.5×10^6 c.p.m., approx. 1 μCi , 28.9 mCi/mmol) was then added. After the reaction had proceeded for 10 min at 30°C , it was terminated by the addition of 10ml of 15% trichloroacetic acid.

Extraction of radioactive lipids

The precipitate that resulted from addition of trichloroacetic acid was collected by centrifugation, and washed twice with 10ml portions of cold 5% (w/v) trichloroacetic acid and once with cold water. The insoluble material was extracted twice with 16ml portions of 45% (w/v) phenol at 0°C for 10 min, with stirring. The phenolic and aqueous phases were

separated by centrifugation, and the phenolic phases were combined and washed with 10ml of water. The phenolic phase was dialysed at 4°C against successive 1000ml volumes of water until the phenol had been removed from the dialysis bag. The contents of the dialysis bag included a brown precipitate, which was collected by centrifugation and washed with water. The precipitate was extracted once with a 20ml portion and twice with 10ml portions of butan-1-ol-6M-pyridinium acetate (pH4.3)-water (2:1:3, by vol.). The phases were separated by centrifugation, and the butanol phases were combined and washed with 10ml of water. The butanol extract was evaporated to dryness under reduced pressure, and the residue dissolved in a small volume of chloroform-methanol (1:1).

Preparation of lipid from P. aureus

About 3.5kg of 4-day *P. aureus* seedlings were homogenized in 100g batches with 300ml of chloroform-methanol (1:1, v/v). Initial homogenization was for 0.5min in a Waring Blendor followed by 5min at high speed in a VirTis 45 homogenizer. The insoluble residue was collected by filtration and re-extracted, with stirring overnight, with 5 litres of chloroform-methanol (2:1, v/v), and the insoluble residue again removed by filtration. The filtrates from the two extractions were combined, placed in a separatory funnel and the chloroform phase was collected. The chloroform extract was washed with 0.25 vol. of 3M-KCl, the aqueous phase and precipitated material were separated and the chloroform phase was collected. The chloroform extract was evaporated to dryness under reduced pressure, and the crude lipid residue stored at -20°C.

Purification of radioactive lipids

A preliminary separation of the radioactive lipids was performed by batch elution of a 1.2cm × 15cm BioSil A silicic acid column (Fig. 1). The radioactivity appeared in two major fractions. The less-polar radioactive lipids eluted in the first two fractions were termed lipid A; the more polar, which appeared in the chloroform-methanol (1:1, v/v) fraction, were termed lipid B.

A preliminary fractionation of 1g of crude *P. aureus* lipids, prepared as described above, was performed by batch elution of a 25cm × 2.5cm BioSil A column. The column was eluted sequentially with 800ml volumes of hexane, hexane-chloroform (3:1, v/v), hexane-chloroform (1:1, v/v), hexane-chloroform (1:3, v/v), chloroform, chloroform-methanol (3:1, v/v) and chloroform-methanol (1:1, v/v). T.l.c. in three solvent systems of samples of each fraction indicated that fraction 2 [hexane-chloroform (3:1, v/v)] and fraction 3 [hexane-chloroform (1:1, v/v)]

contained the only lipid materials with mobilities similar to those of radioactive lipid A. Therefore radioactive lipid A was mixed with the lipids obtained from these fractions, and this mixture was further purified as described below.

With a 1.2cm × 60cm BioSil A silicic acid column, by elution with a single solvent mixture and collection of relatively small samples, the mixture was separated into three radioactive components, each of which moved as a single substituent on t.l.c. Two prior column-chromatographic runs, with hexane-chloroform (1:1, v/v) and benzene-methanol (19:1, v/v) as eluting solvents, removed non-radioactive impurities but the radioactive material was eluted in one peak in each case. With benzene as an eluting solvent and collection of 1ml fractions, the radioactive material was separated into three distinct components (termed lipid A-5, A-7 and A-9), which appeared to be radiochemically and chemically pure.

Kuhn-Roth degradation

Lipid A-5 was oxidized with 2ml of conc. H₂SO₄ and 5ml of 2.5M-chromic acid according to the procedure described by Ginger (1944). After refluxing for 90min the solution was cooled and excess of chromate reduced with hydrazine hydrate. The solution was distilled to a small volume, 5ml of water added, and the distillation repeated twice. The distillate was diluted to 25ml and radioactivity determined by means of liquid-scintillation counting.

Spectroscopy

N.m.r. spectroscopy was performed by using a Varian HA-100 or A-60 spectrometer, infrared spectroscopy with a Perkin-Elmer 621 spectrometer, and mass spectroscopy with a Hitachi Perkin-Elmer RMU-6E spectrometer. All of the spectroscopy was performed by Mrs. Vicki Gindelsperger. Specific conditions are listed in the legends to figures.

Thin-layer chromatography

The t.l.c. plates were prepared with a Brinkmann-DeSaga spreader in 0.25mm thickness by using silica gel H. The chromatograms were allowed to develop until the solvent front had moved 10cm. Radioactivity was localized by removing 1cm sequential sections of the layer, suspending the powder in counting solvent and determining radioactivity with a liquid-scintillation spectrometer. Lipids were localized by spraying the chromatogram with either 0.2% 2',7'-dichloro-fluorescein in ethanol or 0.05% Rhodamine B in ethanol, and illuminating with u.v. light. Solvents used for development in the purity criteria were: benzene-methanol (19:1, v/v); chloroform; hexane-

chloroform (1:1, v/v); benzene; benzene-chloroform (1:1, v/v); benzene-dioxan-acetic acid (90:25:4, by vol.); benzene-methanol-acetic acid (45:8:4, by vol.); benzene-ethyl acetate (19:1, v/v); chloroform-methanol-water (12:6:1, by vol.). The best separation of lipids A-5, A-7 and A-9 was obtained by triple development with benzene.

Results

Incubation of particulate preparations derived from *P. aureus* seedlings with *S*-adenosyl[*Me*- ^{14}C]-methionine results in the labelling of lipid material. With the reaction conditions that are described in this report, more than 10% of the added radioactivity was incorporated into lipids. Special attention must be paid to the extraction of the radioactive lipids that result from the incubation of *S*-adenosyl[*Me*- ^{14}C]-methionine with this enzyme preparation. Very poor yields resulted from extraction of the reaction mixture with typical organic solvents. These poor yields may be a consequence of (1) the inaccessibility of the radioactive lipids to the extracting solvents, (2) a strong interaction between the lipids and polymeric materials in the enzyme preparation or (3) the lipids being covalently bonded to substances that are only slightly soluble in apolar solvents, and becoming soluble on mild acid treatment. The best yields were obtained by extracting the reaction mixture with an aq. 45% phenol emulsion. The lipid radioactivity dissolves in the phenol phase and insoluble, as well as water-soluble, polysaccharide and other polymeric material can be removed. On replacing the phenol with water by dialysis, the radioactive lipids precipitated along with protein. Extraction of this precipitate with polar organic solvents dissolved the radioactive lipids, but even better extraction resulted if the precipitate was suspended in a concentrated salt solution or dispersed by sonic oscillation.

A preliminary silicic acid-column chromatographic procedure separated the radioactive lipids into apolar and polar fractions, designated lipid A and lipid B (Fig. 1). Lipid A was resolved into three radioactive components and purified by repeated silicic acid chromatography on tall columns with apolar solvents. These radioactive components were designated lipid A-5, A-7 and A-9, the numbers reflecting the relative order of elution from the column. The radioactive lipids appeared to be pure, as examined by t.l.c. in a number of solvent systems (see the Methods section). Very small quantities of the purified materials were available. Lipid A-5 was the only component isolated in sufficient quantity to allow elucidation of its structure.

Structure of lipid A-5

The mass spectrum of lipid A-5 appears in Fig. 2. The spectrum is characterized by a series of alkane-

type fragments at m/e 43, 57 and 71–155, representing $[\text{C}_{11}\text{H}_{23}]^+$, and 197–365 representing $[\text{C}_{26}\text{H}_{53}]^+$. These are the most prominent ions in the m/e 211–365 region. Also, there are olefin-type fragments at m/e 41, 55, 69 and 83–195, representing $[\text{C}_{14}\text{H}_{27}]^+$. These are the most prominent ions in the m/e 83–195 region. These alkane and alkene ions decrease in intensity with increasing molecular weight. The molecular ion appears at m/e 410, and the presence of $[M-18]^+$ and $[M-31]^+$ fragments indicates the presence of a hydroxyl group. There is also a mass peak at m/e 31, representing $[\text{CH}_2=\text{OH}]^+$. There are no ions at $[M-43]^+$ or $[M-61]^+$, indicating that any methyl groups present in the molecule are not located on the carbon atom adjacent to the methyl end of the molecule. The distribution and relative intensities of the fragments (Friedel *et al.*, 1956) indicate that the compound is a primary alcohol with a molecular formula $\text{C}_{28}\text{H}_{57}\text{OH}$. Infrared spectroscopy confirms

Fig. 1. Preliminary fractionation of radioactive lipids

A 1.2cm × 15cm column of silicic acid (BioSil A 100–200 mesh; Calbiochem, Los Angeles, Calif., U.S.A.) was loaded with approx. 200000 c.p.m. of crude lipid. The column was eluted with 80ml volumes of the solvent mixtures indicated on the horizontal axis: (1) hexane; (2) 50% chloroform in hexane; (3) 67% chloroform in hexane; (4) chloroform; (5) 50% methanol in chloroform; (6) methanol.

Fig. 2. Mass spectrum of purified lipid A-5

The mass spectrum was obtained from a Hitachi Perkin-Elmer RMU-6E mass spectrometer operating at 80eV, an ionization current of 10 μ A and an ion-source temperature of 250°C.

the presence of a hydroxyl group with absorptions at 3300 and 3400 cm^{-1} due to O-H stretching, and a C-O stretching absorption at 1055 cm^{-1} typical of a primary alcohol.

The n.m.r. spectrum of lipid A-5 appears in Fig. 3 and is characterized by its extreme simplicity. The principal features of the spectrum are a multiplet at τ 9.10, representing methyl proton absorbance, a strong singlet at τ 8.76, representing methylene proton absorbance, and a triplet at τ 6.36, representing absorbance of protons on the carbon atom α to the hydroxyl group. With the exception of the absorption of the hydroxyl proton at τ 8.28, the chemical shifts and splitting patterns for this spectrum are in exact agreement with those found for *n*-decanol (Varian NMR Spectra Catalog, 1962), and for dodecyl alcohol (Catalog of NMR Spectrograms, 1967). The absorption position for the hydroxyl proton would be expected to occur in different positions, as a function of differences in concentration and purity. Integration of the lipid A-5 n.m.r. peaks at τ 8.76 and 9.10 results in a ratio of about 8.0. For a primary alcohol with a molecular formula $\text{C}_{28}\text{H}_{57}\text{OH}$, this ratio represents 6 methyl protons and 48 methylene protons, or a straight-chain alcohol with one methyl branch. The n.m.r. spectra and i.r. spectra contain no evidence of unsaturation, a cyclopropyl ring, nor evidence of any functional groups other than a primary alcohol, methyl groups and methylene groups.

To determine whether the radioactivity derived from *S*-adenosyl[Me- ^{14}C]methionine was localized in the methyl group of the molecule, an amount equivalent to 5788 c.p.m. was subjected to a Kuhn-Roth degradation. The distillate contained 3950 c.p.m. or 68% of the original radioactivity. This yield falls within the expected recovery range for acetic acid derived from methyl groups in the molecule.

Discussion

The small quantity of the radioactive lipids that was available eliminated most techniques of structure elucidation. Except for the simplicity of structure exhibited by one of the radioactive lipids, only a partial characterization would have been possible. The infrared spectra of lipid A-5 indicated that a large percentage of the molecule consisted of a hydrocarbon chain, since those absorbances associated with C-H bond stretching and deformation were by far the major peaks. The only indication of a functional group in the infrared spectrum of lipid A-5 was relatively minor absorbance at wavelengths typical of primary hydroxyl groups. The n.m.r. spectrum (Fig. 3) confirmed this indication, since absorbances at positions associated with methylene protons, methyl protons, methylene protons α to a hydroxyl group, and hydroxyl protons were the principal features of

Fig. 3. Nuclear magnetic resonance spectrum of lipid A-5

The spectrum was obtained from a Varian HA-100 spectrometer with a sample concentration of approx. 5% in C^2HCl_3 . The solution contained 1% tetramethylsilane, with the proton absorbance for this compound set at $\tau = 10$.

the spectrum. Also, the large relative intensity of the methylene proton absorbance indicated that the main feature of the molecular structure was a long hydrocarbon chain. Comparison of the chemical shifts and splitting patterns for lipid A-5 and those of two known primary alcohols, *n*-decanol (Varian NMR Spectra Catalog, 1962) and *n*-dodecanol (Catalog of NMR Spectrograms, 1967) indicates that the primary features of the three spectra are essentially superimposable. The only notable difference is the relative intensity of the absorbances, with the relative intensity of the lipid A-5 methylene proton absorbance being considerably larger than that of decanol or dodecanol. Consequently, lipid A-5 must have a longer hydrocarbon chain than either decanol or dodecanol. The mass spectrum of lipid A-5 (Fig. 2) indicated a molecular weight of 410, as well as providing further evidence confirming that lipid A-5 was a long-chain saturated primary alcohol. The distribution and relative intensities of the charged fragments are those expected of a long-chain primary alcohol (Friedel *et al.*, 1956). Comparison of the lipid A-5 mass spectrum with mass spectra of C_{20} , C_{22} , C_{24} ,

C_{26} , C_{27} and C_{29} straight-chain primary alcohols (Mass Spectrometry Data Centre, spectra nos. MSDC 403–MSDC 408) confirms the prediction of distribution and relative intensities of fragments produced from long-chain alcohols. The ratio of methylene proton absorbance to methyl proton absorbance in the n.m.r. spectrum of lipid A-5 (Fig. 3) is approx. 8. From the molecular weight of 410 determined by mass spectroscopy, this ratio represents 6 methyl protons and 48 methylene protons. The methylene proton absorbance does not include a contribution from the methylene protons on the carbon atom α to the hydroxyl group, or from the protons on carbon atoms bonded to methyl groups in the chain. Therefore lipid A-5 can have only one methyl branch in the hydrocarbon chain. Although there are several possibilities suggested by the mass spectra of lipid A-5, all near the centre of the hydrocarbon chain, it is impossible to determine the position of the methyl branch with the data available. Therefore the structure of lipid A-5 is:

The nature of the enzyme system, and the structure of lipid A-5, indicate that the reaction producing this radioactive lipid was a direct transmethylation. The enzyme preparation was a washed particulate fraction that should contain only minimal quantities of microsomal material. Since no substrates, cofactors or energy sources were provided, it is highly unlikely that this enzyme preparation could catalyse the formation of hydrocarbon chains from breakdown products of *S*-adenosyl[$Me-^{14}C$]methionine. The limited distribution of radioactivity within the carbon chain of the C_{28} alcohol that resulted from one of these reactions supports this conclusion. Therefore the reaction producing radioactive lipid A-5 is probably one utilizing *S*-adenosyl[$Me-^{14}C$]methionine as a methyl group donor and, by analogy with bacterial enzyme systems (Akamatsu & Law, 1970), an unsaturated C_{27} alcohol,

(or an acid-labile derivative of this compound), as a methyl group acceptor. To prove this the methyl group acceptor must, of course, be isolated and characterized.

This work was supported by U.S. Atomic Energy Commission Contract no. AT-(11-1)-2071.

References

- Akamatsu, Y. & Law, J. H. (1968) *Biochem. Biophys. Res. Commun.* **33**, 172
- Akamatsu, Y. & Law, J. H. (1970) *J. Biol. Chem.* **245**, 701

- Alexander, G. J. & Schwenk, E. (1958) *J. Biol. Chem.* **232**, 611
- Bonner, J. & Varner, J. E. (eds.) (1965) *Plant Biochemistry*, pp. 636, 696, 736, Academic Press, New York
- Catalog of NMR Spectrograms (1967) spectrum no. 103M, Sadtler Research Laboratories, Philadelphia
- Chung, A. E. & Goldfine, H. (1965) *Nature (London)* **206**, 1253
- Friedel, R. A., Schultz, J. L. & Sharkey, A. G., Jr. (1956) *Anal. Chem.* **28**, 926.
- Ginger, L. G. (1944) *J. Biol. Chem.* **156**, 453
- Kaneda, T. (1968) *Biochemistry* **7**, 1194
- Kauss, H. & Hassid, W. Z. (1967) *J. Biol. Chem.* **242**, 3449
- Law, J. H. (1967) in *The Specificity of Cell Surfaces* (Davis, B. D. & Warren, L., eds.), p. 87, Prentice-Hall, Englewood Cliffs
- Mass Spectrometry Data Centre, Atomic Weapons Research Establishment, Aldermaston, Berks., U.K., spectra nos. MSDC 403, 404, 405, 406, 407 and 408
- Polacheck, J. W., Tropp, B. E., Law, J. H. & McCloskey, J. A. (1966) *J. Biol. Chem.* **241**, 3362
- Thomas, P. J. & Law, J. H. (1966) *J. Biol. Chem.* **241**, 5013
- Varian NMR Spectra Catalog (1962) vol. 1, spectra nos. 281, 282, Nation Press, Palo Alto
- Villemez, C. L., Swanson, A. L. & Hassid, W. Z. (1966) *Arch. Biochem. Biophys.* **116**, 446