Biosynthesis of Tuberculostearic Acid in a Cell-free Extract. Identification of 10-Methylenestearic Acid as an Intermediate

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Two different types of biological *C*-methylation reactions have been described recently: in the one, all three deuterium atoms of [*C*-$^2H_3$]methionine are retained in the *C*-methylated product; in the other, only two of the deuterium atoms are retained.[1,2] The first type is operative when strongly nucleophilic double bonds are methylated, leading to *C*-methylated products such as thymine riboside,[3] mycophenolic acid,[4] sclerotiorin,[5] dihydromenaquinone-9.[5]

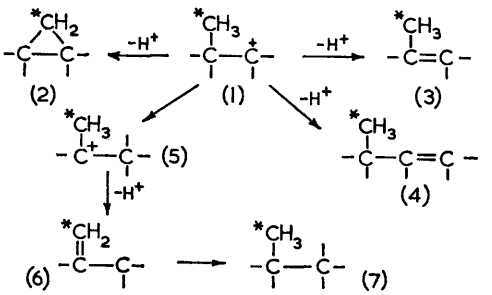
The transfer of only two hydrogen atoms has been observed for tuberculostearic acid and for ergosterol biosynthesis;[1,2] in both cases the precursor has an aliphatic, non-activated double bond. We have, however, reported recently[5] that in the biosynthesis of α-smegmamycolic acid[6] the whole methyl group is transferred to an aliphatic double bond, with migration of the latter (reaction 1 → 4 Scheme*); see also reference 7.

This lead us to postulate that in the two above mentioned cases where one H atom is lost during *C*-methylation, a methylene compound must be an intermediate[5] (reactions 1 → 5 → 6, → 7, Scheme).

We have also shown[10] that during the biosynthesis of tuberculostearic acid (IIIa) a hydrogen

* This scheme, analogous to those of Nes[8] and Clayton[9], was suggested in a recent paper.[5]

migration occurs (from C-10 to C-9), in agreement with the intermediate formation of a methylene compound (IIa).



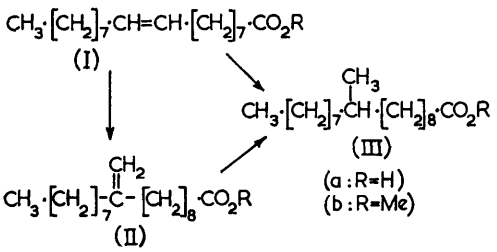
Scheme. Showing the different possible end products derived from the carbo-cation (1) considered to be the first stage of *C*-methylation.

In the steroid field, natural 24-methylene compounds are known (*e.g.*, 24-methylenecholesterol,[11] eburicoic acid,[12] etc.) and quite recently, Akhtar *et al.*,[13] and Barton *et al.*,[14] have shown that in ergosterol biosynthesis by yeast a 24-methylene compound is an intermediate.

These papers prompt us to report our experiments showing that the as yet unknown 10-methylenestearic acid (IIa), is in fact, an intermediate in the biosynthesis of tuberculostearic acid (IIIa) produced by *C*-methylation of oleic acid (Ia) in the presence of methionine.

Our experiments were performed with cell-free extracts of *M. phlei*.[15] After incubation of these extracts (90 ml.) with [*Me*-^{14}C]methionine ($1{\cdot}1 \times 10^8$ d.p.m.) for 3 hours at 30°, hydrolysis, extraction of the acids ($9{\cdot}76 \times 10^6$ d.p.m., 8·8% incorporation), isolation of the methanol-soluble fraction, and esterification with diazomethane, vapour-phase chromatography with a gas radiochromatography-counting system (Nuclear Chicago) on diethylene glycol succinate (DEGS) (20%, 4 m. at 180°, N_2, 20 ml./min.) showed two major peaks of radioactivity having the same retention times as authentic samples of (IIIb) (16 min.) and (IIb) (21 min.), respectively. Identity of retention times was also observed on SE 30, 10% (at 200°). A Kuhn–Roth oxidation of the former fraction (IIIb) ($4{\cdot}7 \times 10^3$ d.p.m., 4 mg.) gave 60% of the radioactivity in acetic acid ($2{\cdot}8 \times 10^3$ d.p.m.) and 7% in CO_2 ($3{\cdot}5 \times 10^2$ d.p.m.).†

Treatment of an aliquot of these esters ($3{\cdot}06 \times 10^5$ d.p.m.) by mercuric acetate[16] allowed the separation of the saturated esters ($1{\cdot}05 \times 10^5$ d.p.m.) from the unsaturated one.‡ This last fraction ($1{\cdot}06 \times 10^5$ d.p.m.) analysed by the gas radiochromatography-counting system on the DEGS 20% column showed one radioactive peak having the same retention time as synthetic methyl 10-methylenestearate (IIb);[17] hydrogenation of this fraction (PtO_2–methanol) gave a radioactive compound having the same retention time as methyl tuberculostearate (IIIb). A Kuhn–Roth oxidation of the hydrogenated fraction gave 55% of the radioactivity in acetic acid and 8% in CO_2.



Ozonisation of (IIb) gave a radioactive volatile fraction, the 2,4-dinitrophenylhydrazone of which had the same R_f value on t.l.c. as authentic 2,4-dinitrophenylhydrazone of formaldehyde.

In another experiment synthetic 10-methylene-[^{14}C]stearic acid[17] ($5{\cdot}6 \times 10^6$ d.p.m., 11·5 mg.) was incubated three weeks with whole cells of *M. phlei* (2 l. of culture medium).[18] After extraction, esterification with diazomethane and elimination of the unsaturated fatty esters by mercuric acetate, we obtained a radioactive fraction ($5{\cdot}7 \times 10^4$ d.p.m., 13·2 mg.). Vapour-phase chromatography on DEGS 20% at 180° showed the coincidence of the radioactivity with the peak of authentic methyl tuberculostearate. After purification by g.l.c. we obtained pure methyl tuberculostearate (IIIb) ($8{\cdot}5 \times 10^3$ d.p.m., 1·7 mg.).

A Kuhn–Roth oxidation of (IIIb) ($7{\cdot}2 \times 10^3$ d.p.m., 1·4 mg.) gave 67% of the radioactivity in acetic acid ($4{\cdot}8 \times 10^3$ d.p.m.), none in CO_2.

These results, establishing the sequence (Ia) → (IIa) → (IIIa) as well as those reported earlier

† We have checked with authentic (IIIb) that the yield of the Kuhn–Roth oxidation under the conditions used is 65 to 70%.

‡ We have not yet found a satisfactory stationary phase to separate cleanly methyl 10-methylenestearate from methyl 9,10-methylenestearate (dihydrosterculate), another possible intermediate.[2] However, we have checked that the cyclopropane ester behaves like the saturated esters in the mercuric acetate precipitation.

by Akhtar *et al.*,[13] and by Barton *et al.*,[14] explain why in some cases,[1] only two of the three hydrogens of the methyl group of methionine are retained in the *C*-methylated product.

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