ENZYMATIC SYNTHESIS OF 10-METHYLENE STEARIC ACID AND TUBERCULOSTEARIC ACID*

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Tuberculostearic acid (10-methylstearic acid) is a major component of the phospholipid fatty acids of Mycobacterium species. Whole cell experiments by Lennarz, et al. (1962), and by Jaureguiberry, et al. (1965), demonstrated that the methyl carbon at 10, and two of the methyl protons arose from the methyl group of methionine. Jaureguiberry, et al. (1966), demonstrated that extracts of M. phlei converted (methyl-^{14}C)-methionine to labeled 10-methylene stearic acid and 10-methylstearic acid derivatives. We have obtained evidence for the following details of this enzymatic sequence:

1) Oleyl phospholipid + S-adenosylmethionine $\longrightarrow$ 10-methylene stearyl phospholipid + S-adenosyl homocysteine

2) 10-Methylene stearyl phospholipid + NADPH $\xrightarrow{+H^+}$ 10-methylstearyl phospholipid + NADP

Enzymatic extracts of M. phlei were prepared from cells grown in Sauton medium according to Azerad et al. (1965). Labeled fatty acids were isolated from incubation mixtures after saponification of the lipid products. These labeled free fatty acids were first examined on a silicic acid thin layer chromatogram by using two solvent systems: $CHCl_3$-MeOH-H_2O (75:15:1) and petroleum ether-ether-acetic acid (60:40:1). They were then converted to methyl esters by treatment with diazomethane, and examined by thin layer chromatography (TLC) on silicic acid impregnated with $AgNO_3$ for separation of olefinic and saturated acid esters and by gas chromatography (GLC) for resolution into individual fatty acid esters. Using OV-17 (Supelco) as the stationary phase we were able to resolve cleanly the methyl esters of tuberculostearic, 10-methylene stearic and C-19

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Table 1

Incorporation of Radioactivity from (Methyl-^{14}C)-S-adenosylmethionine into 10-Methylene Stearic Acid (MSA) and 10 Methylstearic Acid (TSA)

Experiment	Incorporation into FA[1] % of added SAM	Incorporation into MSA %[2]	Incorporation into TSA %[2]
1	8.06	62.7	23.4
2	4.58	68.7	22.7
3	4.51	57.7	35.6

1 In a typical experiment, 200 mμmoles of methyl-^{14}C SAM were added in an incubation mixture which contained 100 μmoles of potassium phosphate (pH 7.04) and the crude enzyme (10 mg protein), total volume 1.1 ml. Incubation was at 30°C for 1 hour. The crude enzyme was obtained by centrifugation, at 100,000 x g for 60 minutes, of sonically disrupted cell suspension of logarithmically growing M. phlei cells.

2 Calculated from radioactivity recovered in gas chromatographic fractions. Recovery from GLC averaged 70-80%. In order to confirm the radioactivity corresponding to 10-methylene stearate, the labeled ester was collected from GLC and subjected to hydrogenation in the presence of Pt/H_2. After hydrogenation, the ester was reapplied to GLC and the radioactivity was found exclusively in the tuberculostearate fraction.

cyclopropane fatty acids (retention times, relative to methyl stearate = 1: 1.14, 1.33, 1.45 respectively). At no time did we observe incorporation of label into the cyclopropane fatty acid fraction. Table 1 indicates the incorporation of label from methyl-^{14}C S-adenosyl-methionine (SAM) into 10-methylene stearic acid and tuberculostearic acid.

In order to find out what reducing agent supplied hydrogen for the conversion of 10-methylene stearate to tuberculostearate, we treated enzyme extracts with activated charcoal. Such treatment abolished the synthesis of tuberculostearic acid but permitted synthesis of 10-methylene stearic acid (see Table 2). We then supplemented the treated extracts with either a NADH or NADPH generating system. Supply of an adequate amount of NADPH permitted the resumption of tuberculostearic acid synthesis while NADH was without effect (Table 2).

None of these experiments indicated what derivative of oleic acid was undergoing reaction since all products were examined after saponification of all of the esters. In order to investigate this question we incubated with methyl-^{14}C SAM and extracted the lipids with one of the following: $CHCl_3$-MeOH (2:1), $CHCl_3$-MeOH-H_2O (40:15:2.5) (Lee and

Table 2

Effect of Cofactors on the Incorporation of Radioactivity into Fatty Acids

Conditions	Incorp. into FA, % of added SAM[1]	Incorp. into MSA, %[2]	Incorp. into TSA, %[2]
Crude enzyme	3.36	68.7	22.7
Charcoal treated enzyme (CTE)[3]	5.27	87.7	2.9
CTE + 1 μmole NADPH	6.31	58.4	30.9
CTE + 3 μmoles NADPH	6.00	57.7	27.0
CTE + generating system without NADP	6.14	69.0	2.3
CTE + NADPH generator + 1 μmole NADP	5.84	44.8	22.3
CTE + NADPH generator + 3 μmoles NADP	5.54	44.2	25.0
CTE + generating system without NAD	2.64	83.9	3.7
CTE + NADH generator + 3 μmoles NAD	3.21	86.3	3.1
CTE + NADH generator + nicotinamide + ergothioneine	1.47	90.0	2.4
CTE + NADH generator + 3 μmoles NAD + nicotinamide + ergothioneine	1.74	76.9	2.2

1 In this experiment, the incubation mixture contained: 140 μmoles of potassium phosphate (pH 7.05), crude enzyme (10 mg protein) or charcoal treated enzyme (CTE)[3], 200 mμmoles of methyl-^{14}C SAM, 1 or 3 μmoles of cofactors, and cofactor generating system in a total volume of 1.5 ml. The NADPH generating system consists of 15 μmoles of glucose-6-phosphate sodium salt and 10 μg of glucose-6-phosphate dehydrogenase (from yeast, specific activity 140 EU/mg protein, Calbiochem). In the case of the NADH generating system, 3 mmoles of ethanol and 1 mg of alcohol dehydrogenase (from horse liver, specific activity 1.4 EU/mg protein, Sigma), 150 μmoles of nicotinamide and 0.1 μmole of ergothioneine were added to inhibit NADase activity (Grossman and Kaplan, 1958). The extracts were incubated at 30°C for 1 hour.

2 Calculated from radioactivity recovered in gas chromatographic fractions. Recovery from GLC averaged 75-90%.

3 3 ml of crude enzyme (protein content 10 mg/ml. See footnote of Table 1) were mixed with 200 mg of Norit A and the mixture was kept standing for 1 hour at 0°C with occasional stirring and then centrifuged twice at 24,000 x g for 15 minutes. The clear supernatant solution thus obtained was used as the charcoal treated enzyme preparation (CTE).

Ballou, 1964), or boiling methanol (Ballou, et al., 1963) prior to saponification. The three extraction methods gave essentially the same result. The labeled lipids were chromatographed on silicic acid TLC. Individual labeled lipids were isolated and saponified. The fatty acids recovered from each fraction were examined by TLC and GLC. It was found that essentially all of the labeled tuberculostearic and 10-methylene stearic acid were components of phospholipid fractions - phosphatidylinositol oligomannosides and phosphatidylethanolamine - which were identified by two-dimensional TLC (see Table 3). No

Table 3

Radioactivity Distribution Among Individual Lipids on a Silicic Acid Thin Layer Chromatogram

Spot No.		% of total radioactivity[1]	Radioactivity in FA[3] %	Radioactivity FA in spot/total FA %		Radioactivity[4] distribution among phospholipids on 2-D TLC, %
1		0.2	0	0		
2		1.3	0	0		
3		8.7	0	0		
4		2.8	0	0		
5		6.2	0	0		
6		2.6	0	0		
7		16.1	0	0		
8		11.1	0	0		
9	CL & PE frac.[2]	6.4	11.5	2.3		PE 44.9
10		11.6	85.9	31.3	52.0	CL 8.6
11		6.7	87.6	18.4		
12		4.8	37.5	5.7		
13	PIM frac.[2]	17.6	66.8	37.0	48.0	PIM 46.5
14		2.7	41.6	3.5		
15		1.2	46.6	1.8		

1 Incubation mixture: 100 µmoles potassium phosphate (pH 7.04), 38 mg crude enzyme protein, 200 mµmoles methyl-^{14}C SAM, total volume 1.1 ml. Incubation at 30°C for 1 hour. Acetone added to stop reaction. Supernatant solution removed and reduced to dryness. Both supernatant fraction and precipitate were extracted with $CHCl_3$-MeOH (2:1) (see text). Crude lipids were washed (Folch, et al., 1957) and applied to TLC (Brinkmann Instrument Co., silica gel). The solvent system $CHCl_3$-MeOH-H_2O (65:25:4) was used.

2 On the thick layer plate used here, no clear separation was achieved between cardiolipin (CL) and phosphatidylethanolamine (PE), nor between individual phosphatidylinositol oligomannosides (PIM).

3 Spots were scraped from the plate and the lipids were saponified prior to fatty acid (FA) isolation. Figures represent CPM in FA/CPM in spot. Recovery of fatty acids compared to the fatty acid content of the applied lipids was over 90%.

4 A portion of the labeled lipid fraction was mixed with carrier M. phlei lipids and subjected to two dimensional TLC on silica gel using $CHCl_3$-MeOH-H_2O (65:25:4) in one direction and $CHCl_3$-MeOH-AcOH-H_2O (60:30:8:4) in the second. This system permitted clean resolution of PE, CL, phosphatidylinositol and their lyso derivatives, but did not resolve phosphatidylinositol from the oligomannoside derivatives.

radioactivity was found in the free fatty acid or lysophosphatide regions of these chromatograms.

These results indicate, but do not prove, that the enzymatic conversions take place on

phospholipid substrates. We have attempted to add exogenous unsaturated phospholipids in order to stimulate the enzymatic reactions, but so far the results have been disappointing. The tuberculostearate synthetase seems to resemble the cyclopropane synthetase of Serratia (Zalkin, et al., 1963) in which only endogenous phospholipids serve as substrates. The possibility that the tuberculostearate synthetase reactions take place on fatty acid derivatives which are later converted to phospholipids still exists, but the fact that 10-methylene stearic acid, an intermediate in the overall reaction, accumulates in phospholipid form, tends to strengthen the evidence for transformation at the phospholipid level.

Brennen and Ballou (1967) have determined the fatty acid content of the phospholipids extracted from M. phlei. They found that tuberculostearic acid constitutes about 40% of the fatty acids of the phosphatidylinositol dimannosides, but only 7% of those of phosphatidylethanolamine and cardiolipin. In our in vitro system we find a similar low amount of alkylated acids in cardiolipin, but both phosphatidylethanolamine and the inositides have an equally high content of labeled acids. It is of interest that the latter two phosphatides, both of which show relatively low turnover, accumulate alkylated acids, while cardiolipin, with a high turnover rate (Akamatsu, et al., 1967) contains only a small amount of alkylated acid.

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