Enzymatic Alklenation of Phospholipid Fatty Acid Chains by Extracts of *Mycobacterium phlei*  

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**SUMMARY**  

The enzymatic synthesis of tuberculostearic acid (10-methylstearic acid) was catalyzed by extracts of *Mycobacterium phlei*. This process involved two reactions of the olefinic fatty acid chain of phospholipids. The chain was first alklenated at the 10-carbon to give a methylene group, which was subsequently reduced to a methyl group. The first reaction could be measured by using S-adenosylmethionine-methyl-14C. The enzyme was found in the supernatant fraction when extracts of cells broken by sonic oscillation were subjected to centrifugation at 100,000 × g. S-Adenosyl-L-methionine was the only effective donor of the 1-carbon unit. Phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine were substrates for the reaction, and both 16- and 18-carbon chains were alklenated although only the 10-olefinic chains appeared to be converted. The enzyme acted upon chains at either position 1 or 2 of the glyceride molecule. Several detergents had little effect on the rate of the reaction.

Tuberculostearic acid (10-methylstearic acid) is a characteristic component of the membrane lipids of the mycobacteria (1, 2). The synthesis of this compound was first studied *in vivo* by Lenaz, Scheuerbrandt, and Bloch (3), who established that the precursors were oleic acid and the methyl group of methionine. Later experiments by Jauréguiberry et al. (4, 5) showed that the transfer of the methionine methyl group occurred with loss of a proton. The reaction was also accompanied by a shift of the olefinic proton at the 10-carbon of oleate to the 9-carbon (6). These facts led to the formulation of a logical mechanism:  

![Reaction Mechanism Diagram](http://www.jbc.org/)

Further evidence for this scheme was produced by Jauréguiberry et al. (7), who isolated labeled 10 methylstearic acid and labeled tuberculostearic acid from a cell homogenate of *Mycobacterium phlei* which had been incubated with methionine-methyl-14C and subsequently saponified. The details of the reaction, including the nature of the endogenous lipid acceptor, the actual methyl donor, and the hydrogen donor for the reduction of the 10-methylene group to a methyl group, remained to be specified. We have reported preliminary evidence that these processes are described by the following reactions in crude, cell-free extracts (8):  

1. (Olefinic fatty acyl) phospholipid + S-adenosylmethionine $\rightarrow$ (methylene acyl) phospholipid + S-adenosylhomocysteine  
2. (Methylene acyl) phospholipid + NADPH + H+ $\rightarrow$ (methyl acyl) phospholipid + NADP+  

In this paper we consider only the first of these reactions, although both enzyme activities were present in our preparation.

**EXPERIMENTAL PROCEDURE**

**Materials**

*M. phlei* ATCC 254 cells were grown at 30° on Sauton medium (9), harvested at middle or late log phase by filtration, and washed with distilled water. Washed cells were stored at -15°. S-Adenosyl-L-methionine-methyl-14C was purchased from New England Nuclear. Unlabeled S-adenosyl-L-methionine was purchased from Calbiochem. Unisil silicic acid was obtained from...
Preparation of Cell-free Extracts by a modified Wittig reaction (12). After about 15 min, this suspension was centrifuged at 12,000 g for 10 min and then twice at 20,000 g for 30 min. The resulting supernatant, designated 20-S was further centrifuged at 105,000 g for 60 min in the Spinco model L preparative centrifuge. This supernatant was adjusted to 10 mg of protein per ml with 0.1 M phosphate buffer (pH 7.0) and disrupted at 0-4° for 10 to 15 min in a sonic disintegrator (Branson Instruments, Inc., Stanford, Connecticut). The disintegrated material was centrifuged at 10,800 × g for 10 min and then twice at 20,000 × g for 30 min. The resulting supernatant, designated 20-S, was further centrifuged at 105,000 × g for 60 min in the Spinco model L preparative centrifuge. This supernatant was adjusted to 10 mg of protein per ml with 0.1 M phosphate buffer (pH 7.0), and designated 100-S. This crude extract (10 mg of protein per ml, 10 ml) was added dropwise to 70 ml of cold acetone (-10 to -15°) with stirring. After about 15 min, this suspension was centrifuged at 12,000 × g for 10 min and the supernatant was discarded. The precipitate was partially dried by subjecting it to water pump vacuum for 20 min in the cold. The wet acetone powder was ground in a chilled mortar to remove the residual acetone and then mixed with 10 ml of 0.1 M phosphate buffer (pH 7.0). The resulting suspension was centrifuged at 27,000 × g for 30 min and the supernatant was removed. The supernatant thus obtained was added to cold acetone and all of the procedures were repeated. Finally, the second acetone precipitate was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0), and a clear yellow supernatant was obtained by centrifuging at 37,000 × g for 60 min. This acetone-treated enzyme preparation, when stored at −10°, retained full activity for at least 1½ months.

Methods

Preparation of Cell-free Extracts

Washed cells (0.2 g per ml) were suspended in 0.1 M phosphate buffer (pH 7.0) and disrupted at 0-4° for 10 to 15 min in a sonic disintegrator (Branson Instruments, Inc., Stanford, Connecticut). The disintegrated material was centrifuged at 10,800 × g for 10 min. The supernatant was removed. The supernatant thus obtained was adjusted to 10 mg of protein per ml with 0.1 M phosphate buffer (pH 7.0), and a clear yellow supernatant was obtained by centrifuging at 37,000 × g for 30 min. After cooling, methanol was removed with a nitrogen stream. The alkaline solutions were acidified to pH 1 by the addition of 6 M HCl. The acidified solutions were extracted three times with 3 ml of ether and the combined ether extracts were washed twice with 2 ml of 2% KCl. Aliquots were taken for radioactivity measurement and for examinations by chromatography on a thin layer of silicic acid. Thin layer chromatography solvent systems were chloroform-methanol-water (75:15:1) or petroleum ether (50-60°)-ether-acetic acid (60:40:1) and the spots corresponding to free fatty acids were detected by radioautography and scraped from the plates into scintillation vials for radioactivity measurement. The methyl esters of the extracted fatty acids were prepared by treatment with diazomethane (13). In order to distinguish methylene fatty acid esters from methyl branched fatty acid esters, 26% silver nitrate-impregnated silicic acid thin layer chromatography was used with the solvent system petroleum ether (30-60°)-ether (9:1). Gas-liquid chromatography was performed on a 4- or 6-foot column, operated at 190-200° with a helium flow rate of 25 to 50 ml per min. Radioactive fatty acid esters were trapped at the outlet of the column in a small capillary tube. For counting, fatty acid esters were rinsed with toluene scintillator fluid from the collection tube into vials.

Preparation of Acetone-treated Enzyme

Crude enzyme preparations generally contained endogenous lipid substrates. For removal of endogenous lipids we tried acetone treatment, butanol extraction, alcohol-ether extraction, and treatment with ammonium sulfate followed by Sephadex column chromatography. Of these, only acetone treatment yielded an enzyme preparation which was significantly free of endogenous lipid substrates and was also active. The crude extract (10 mg of protein per ml, 10 ml) was added dropwise to 70 ml of cold acetone (-10 to -15°) with stirring. After about 15 min, this suspension was centrifuged at 12,000 × g for 10 min and the supernatant was discarded. The precipitate was partially dried by subjecting it to water pump vacuum for 20 min in the cold. The wet acetone powder was ground in a chilled mortar to remove the residual acetone and then mixed with 10 ml of 0.1 M phosphate buffer (pH 7.0). The resulting suspension was centrifuged at 27,000 × g for 30 min and the supernatant was removed. The supernatant thus obtained was added to cold acetone and all of the procedures were repeated. Finally, the second acetone precipitate was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0), and a clear yellow supernatant was obtained by centrifuging at 37,000 × g for 60 min. This acetone-treated enzyme preparation, when stored at −10°, retained full activity for at least 1½ months.

Assay of Enzyme Activity

Method 1—Crude extracts or acetone-treated enzyme were incubated with S-adenosylmethionine-methyl-¹⁴C and other additions as indicated in tables or figures. Most incubations were carried out at 30° for 1 hour. Reactions were stopped by the addition of an equal volume of methanol and 0.2 volume of 85% KOH. These solutions were heated in a boiling water bath for 30 min. After cooling, methanol was removed with a nitrogen stream. The alkaline solutions were acidified to pH 1 by the addition of 6 M HCl. The acidified solutions were extracted three times with 3 ml of ether and the combined ether extracts were washed twice with 2 ml of 2% KCl. Aliquots were taken for radioactivity measurement and for examinations by chromatography on a thin layer of silicic acid. Thin layer chromatography solvent systems were chloroform-methanol-water (75:15:1) or petroleum ether (50-60°)-ether-acetic acid (60:40:1) and the spots corresponding to free fatty acids were detected by radioautography and scraped from the plates into scintillation vials for radioactivity measurement. The methyl esters of the extracted fatty acids were prepared by treatment with diazomethane (13). In order to distinguish methylene fatty acid esters from methyl branched fatty acid esters, 26% silver nitrate-impregnated silicic acid thin layer chromatography was used with the solvent system petroleum ether (30-60°)-ether (9:1). Gas-liquid chromatography was performed on a 4- or 6-foot column, operated at 190-200° with a helium flow rate of 25 to 50 ml per min. Radioactive fatty acid esters were trapped at the outlet of the column in a small capillary tube. For counting, fatty acid esters were rinsed with toluene scintillator fluid from the collection tube into vials.

Method 2—After incubation for appropriate times, reactions were terminated by the addition of 30 ml of chloroform-methanol (2:1) to the incubation mixture. The chloroform-methanol (2:1) solution was mixed with 5 ml of 0.88% KCl and shaken vigorously. The phases were allowed to separate and the aqueous layer was discarded. The organic layer was then washed three times with 10 ml of chloroform-methanol-0.5% KCl (3:48:47). The lower chloroform layer was passed through a sintered glass filter in order to remove the coagulated protein, and the filtrate was evaporated to dryness. The residue was taken up in chloroform-methanol and applied to a silicic acid thin layer chromatography plate. The solvent system was chloroform-methanol-water (65:25:4). Radioactive compounds were detected by scanning with a Nuclear-Chicago Actigraph III, model 1002 strip scanner with model 1006 thin layer chromatography plate scanner attachment, or radioautography. Spots were visualized by exposure to iodine vapors, and the areas corresponding to various lipids were scraped into scintillation vials for counting.

In order to confirm whether the radioactivity in phospholipid spots was truly associated with fatty acyl groups, the compounds were eluted with methanol and hydrolyzed, and the fatty acids were examined by thin layer chromatography and gas-liquid chromatography. Radioautography was performed as described earlier (14). Scintillation counting used a toluene fluid described by Hildebrand and Law (15) with a Packard Tri-Carb model 3000 instrument (Packard Instrument Company, La Grange, Illinois).

It is important to note that in most cases we measured total incorporation into fatty acid derivatives by either assay method.
This is essentially a measure of the first enzymatic step in which the S-adenosylmethionine methyl group is transformed into a fatty acid methyl group. In most cases the second step, reduction to a methyl group, was ignored, although we occasionally report the distribution of radioactivity into both methylenes (unsaturated) and methyl (saturated) fatty acids (e.g. Table III).

Catalytic Hydrogenation of Radioactive 110-Methylene Stearate

Radioactive 10-methylene stearate was collected from the gas-liquid chromatography column and mixed with 1 mg of carrier 10-methylene stearate. This was dissolved in 1 ml of methanol and 2 mg of PtO₂ were added. Hydrogenation was carried out overnight with shaking at 30 psi H₂ pressure. The reaction mixture was filtered through a sintered glass filter and the catalyst was washed with methanol and ether. The combined filtrate was reduced in volume and subjected to gas-liquid chromatography.

Preparation of Lipid Substrates

Lipids from Log Phase Cells of M. phlei—Total lipids were extracted with chloroform-methanol and mixed with 1 mg of carrier 10-methylene stearate. This was dissolved in 1 ml of methanol and 2 mg of PtO₂ were added. Hydrogenation was carried out overnight with shaking at 30 psi H₂ pressure. The reaction mixture was filtered through a sintered glass filter and the catalyst was washed with methanol and ether. The combined filtrate was reduced in volume and subjected to gas-liquid chromatography.

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Analytical Methods

Protein concentration was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard. Phosphorus was determined by the method of Allen (22).

RESULTS

Although we used the procedure reported by Jaureguiberry et al. (7) and Azerad, Bleiler-Hill, and Lederer (23) for preparing homogenates of M. phlei, we used much smaller volumes for incubations and S-adenosylmethionine instead of methionine. We used longer periods of centrifugation at higher speeds (see "Methods") because we found that the preparation used by Jaureguiberry et al. (7) contained many whole bacterial cells. Further centrifugal fractionation showed that most of the enzymatic activity resided in the 100,000 × g supernatant fraction (Table I). Addition of phospholipids to the reactions did not stimulate incorporation into alkylated fatty acids (Table I).

Our first task was to establish the nature of the labeled fatty acid derivatives formed in this system when S-adenosylmethionine served as the substrate. For this purpose we used crude 100,000 × g supernatant fraction with S-adenosylmethionine-methyl-¹⁴C and endogenous lipid acceptors. The fatty acid fraction isolated after saponification was treated with diazomethane and the esters were chromatographed on silver nitrate thin layer chromatography. Most of the radioactive compounds were found in the saturated and unsaturated fatty acid ester fractions (Fig. 1). The unsaturated fraction usually contained most of the activity, although the ratio of the two fractions varied with enzyme preparations and incubation conditions.

The formation of 10-methylene stearic and 10-methylstearic acid bears a strong resemblance to the enzymatic formation of cyclopropane fatty acids, a process also involving reaction of S-adenosylmethionine with olefinic chains of phospholipids (10, 14). Since some of the mycobacteria produce very long chain fatty acids with cyclopropane rings (24, 25), it was important to

Table I

| Incubation mixture | Incorporation into fatty acids |
|--------------------|-------------------------------|
| 20,000 × g supernatant (30-S) | 7.70 |
| 100,000 × g supernatant (100-S) | 5.25 |
| 100,000 × g precipitate (100-P) | 1.81 |
| 20-S + phosphatidylethanolamine | 6.58 |
| 100-S + phosphatidylethanolamine | 4.22 |
| 100-P + phosphatidylethanolamine | 0.55 |

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Fig. 1. Separation of fatty acid products on AgNO₃-SiO₂. Incubation conditions were as described in Experiment 2 of Table II. After hydrolysis, the acids were extracted and converted to methyl esters. Plates were prepared with 25% AgNO₃ in Merck Silica Gel G. The solvent system was petroleum ether (30–60°)–ether (9:1). Spots were located by spraying with 2,4-dichloro-fluorescein and viewing under ultraviolet light. Bands were then scraped from the plate for the radioactivity determination. The figure shows the plate at the bottom, and the level of radioactivity in each area of the plate above. MS, methyl 10-methylene stearate; TS, methyl tuberculostearate.

Establish that the enzymatic products that we isolated were not cyclopropane products, but indeed 10-methylene stearic and 10-methylstearic acids. A gas-liquid chromatographic system was therefore devised which would separate esters of these two compounds from each other and from cyclopropane derivatives of comparable chain length. For this purpose, OV 17 served as an appropriate liquid phase (Fig. 2). Note that both methyl tuberculostearate and methyl 10-methylene stearate were labeled. No label was found in the cyclopropane fatty acid ester fraction during these studies. In order to characterize the labeled product which chromateded with carrier methyl 10-methylene stearate, the material was isolated from the column and reduced with hydrogen and platinum in methanol. These conditions do not reduce cyclopropane fatty acid esters. All of the methylene compound was converted to labeled methyl 10-methyl stearate, as judged by rechromatography (Fig. 2).

Early experiments with added exogenous lipids gave disappointing results (Table I), for inhibition usually resulted. An indirect approach was therefore used to obtain information about the endogenous lipid substrate. Following incubation with labeled S-adenosylmethionine, the enzymatic reaction mixture was extracted with solvents and the labeled lipid products were chromatographed on thin layer chromatography. The lipid fractions were isolated and saponified, and the fatty acids were extracted and counted. The results provided a clear
TABLE II

Stimulation of labeled fatty acid synthesis by phospholipids

For Experiment 1, each reaction mixture contained in a final volume of 1.5 ml: methyl-$^{14}$C-S-adenosylmethionine, 1 μCi, 200 μmoles; potassium phosphate (pH 7.0), 140 μmoles; acetone-treated enzymes (4.4 mg of protein). 100-S (protein content, 3 mg) was heated at 100° for 10 min and used as lipid substrate source. Crude lipids (1.5 mg) were extracted and prepared as described under "Experimental Procedure." Products were assayed by Method 1. For Experiment 2, reaction mixture consisted of crude extract (100-S, 10 μg of protein) or acetone-treated enzyme (5.4 mg of protein); methyl-$^{14}$C-S-adenosylmethionine, 0.4 μCi, 100 μmoles; potassium phosphate (pH 7.0); 100 μmoles in a final volume of 2.1 ml. Phosphatidylethanolamine from A. agilis (2 mg) was added as lipid substrate. Other conditions were as described in Experiment 1. For Experiment 3, incubation mixture contained 200 μmoles (0.8 μCi) of S-adenosylmethionine-methyl-$^{14}$C; 200 μmoles of potassium phosphate (pH 7.0); 2 mg of phospholipids as indicated; and acetone-treated enzyme (4.7 mg of protein). Total 2.1 ml. After incubation for 1 hour at 30°, products were isolated and examined by Method 2 (Experimental Procedure).

| Incubation                                                                 | Incorporation into fatty acids (μCi/ hr) |
|---------------------------------------------------------------------------|----------------------------------------|
| Experiment 1                                                              |                                        |
| Acetone-treated enzyme                                                   | 0.17                                   |
| Acetone-treated enzyme + heated 100,000 X g supernatant                   | 0.76                                   |
| Acetone-treated enzyme + crude lipids from log phase cells               | 0.41                                   |
| Experiment 2                                                              |                                        |
| Crude extract                                                             | 1.69                                   |
| Acetone-treated enzyme                                                   | 0.01                                   |
| Acetone-treated enzyme + phosphatidylethanolamine (Fleischer and Klouwen dispersion) | 0.83                                   |
| Experiment 3                                                              |                                        |
| Acetone-treated enzyme                                                   | 0.13                                   |
| Acetone-treated enzyme + phosphatidylethanolamine (Fleischer and Klouwen dispersion) | 0.22                                   |
| Acetone-treated enzyme + phosphatidylethanolamine (sonic dispersion)      | 0.33                                   |
| Acetone-treated enzyme + phosphatidyiglycerol (sonic dispersion)          | 2.01                                   |
| Acetone-treated enzyme + phosphatidylinositol (sonic dispersion)          | 1.77                                   |
| Acetone-treated enzyme + cardiolipin (sonic dispersion)                   | 0.18                                   |

Fig. 3. Products of the enzymatic incubation. Lipids extracted from incubation mixtures as shown in Table II and chromatographed by thin layer chromatograph. After locating the spots with $I_2$, the plates were passed through a plate scanner to locate radioactive compounds. The plates are shown below the scanner record. The origins are on the left and the solvent fronts on the right. The strongest spots in each case are the phospholipid used as substrate, to the left, and carrier methyl oleate, to the right. Top, phosphatidylethanolamine (A. agilis) as substrate; middle, phosphatidylinositol (yeast) as substrate; bottom, phosphatidylglycerol (A. agilis) as substrate.
TABLE III

Labeled fatty acids from phospholipid products

Experimental conditions were as described in Experiments 2 and 3 of Table II. PE, phosphatidylycholine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

| Phospholipid substrate | Phospholipid product | Fatty acids from hydrolyzed phospholipid |
|------------------------|----------------------|----------------------------------------|
|                        |                      | Total C 17 | Total C 19 |
|                        |                      | Unsaturated | Saturated |
|                        |                      | Unsaturated | Saturated |

| Compound | Composition | Total CPM activity | Incorporation into fatty acids by enzyme plus phosphatidylglycerol | Incorporation of fatty acids by enzyme plus phosphatidylglycerol |
|----------|-------------|---------------------|-------------------------------------------------------------------|------------------------------------------------------------------|
| 16:1     | 18:1        | %                   | cpm | mmoles/hr | mmoles/hr |
| PE       | 45a 19a     | PE                  | 4,930 | 4,460 | 3,110 | 1,445 | 1,673 | 140 | 130  |
| PI       | 25a 25a     | PI                  | 10,600 | 6,712 | 3,417 | ND | ND | 2,420 | 2,330 |
| PG       | 33a 12b     | PG                  | 3,550 | 3,282 | 3,092 | ND | ND | 34 | 34  |

* From A. agilis, analysis of Hildebrand and Law (15), 18:1 is cis-vaccenic acid.
† Determined by gas-liquid chromatography. Phosphatidylinositol, from yeast, has oleic acid; phosphatidylglycerol, from A. agilis, has cis-vaccenic acid; M. phlei endogenous lipids contain oleic acid.
‡ See Akamatsu and Law (8).
§ ND, not determined.

TABLE IV

Comparison of effectiveness of various C-1 donors

Each reaction mixture contained: labeled substrate, 200 mmoles (0.8 to 0.86 μCi); acetone-treated enzyme, 4.7 mg of protein; potassium phosphate (pH 7.0), 200 mmoles; and phospholipid, when added, 2 mg. Total volume was 2.1 ml, incubated for 15 min at 30°C. Products were assayed by Method 1 ("Experimental Procedure").

| Labeled substrate | Incorporation into fatty acids by enzyme alone | Incorporation of fatty acids by enzyme plus phosphatidylglycerol |
|-------------------|-----------------------------------------------|-----------------------------------------------------------------|
| S-Adenosylmethionine-methyl-14C | 0.55 mmoles/hr | 4.37 mmoles/hr |
| Methionine-methyl-14C | 0.002 mmoles/hr | 0.07 mmoles/hr |
| Serine-3-14C | 0.04 mmoles/hr | 0.07 mmoles/hr |
| Sodium formate-14C | 0.02 mmoles/hr | 0.01 mmoles/hr |

* From yeast.

indication that both 10-methylene stearic acid and 10-methylstearic acid formed in the enzymatic reaction were bound to phospholipid molecules (8). The results were somewhat complicated by the formation of large amounts of other labeled products, one of which ultimately was proved to be a labeled fatty acid methyl ester formed by alkylation of the fatty acid carbonyl group (20).

A more direct approach became feasible when we obtained an acetone-treated preparation which was relatively free of endogenous lipid substrates. Synthesis of labeled fatty acid derivatives now became dependent upon addition of the extracted lipid fractions or purified phospholipids (Table II). Addition of various phospholipids stimulated the formation of labeled fatty acid derivatives (Table II), but fatty acid methyl esters were also formed (Fig. 3). This was probably the result of the liberation of free fatty acid by the action of phospholipases, followed by carboxyl alkylation catalyzed by fatty acid methyl ester synthetase (26). Different acetone-treated enzyme preparations gave somewhat different results with regard to their response to added phospholipids, to the level of activity which survived acetone treatment, and to the ratio of saturated and unsaturated acid products. Table II shows results with three typical preparations.

Depending upon the nature of the fatty acid chains in the added phospholipid substrates, labeled products of differing chain lengths were synthesized. Thus, when a phosphatidylethanolamine from A. agilis, which contained a large proportion of palmitoleic acid (Table III) was added, labeled products were formed with retention times on gas-liquid chromatography corresponding to 17-carbon acid esters. Lennarz, Scheuerbrandt, and Bloch (3) had observed the formation of a 17-carbon homologue of tuberculostearic acid in M. phlei. Very little 19-carbon product was formed when phospholipids from A. agilis were used, probably because cis-vaccenic acid rather than oleic acid is present (27). Table III summarizes the products observed with various phospholipids.

Because of the report of Jauréguiuberry et al. (7) in which methionine-methyl-14C was used as a substrate, we decided to test various possible C-1 donors for the synthesis of fatty acid products in our enzyme preparations. The data of Table IV show that only S-adenosylmethionine was an effective substrate for this reaction.

Several parameters for the enzymatic reaction were examined. Although no extensive determination of this effect of pH variation has been made, the reaction had about twice the initial velocity at pH 7 as at pH 8. The reaction rate was linear with protein concentration over a 4-fold range, and with time up to 1 hour under the usual conditions of incubation. The effect of the following detergents was tested: sodium dodecyl sulfate, Triton X-100, Tween 80, Cutsicum, and hexadecyltrimethyl ammonium bromide. None had any dramatic stimulatory effect on the rate of the reaction.

The question of positional specificity for olefinic chains at position 1 or 2 on the phosphatide was examined by isolating the fatty acids from each position after the enzymatic alkylation of phosphatidylglycerol and phosphatidylinositol fatty acids.
Positional distribution of enzymatically alkylenated fatty acid chains in phospholipids

| Phospholipid                  | Distribution of olefinic acids in substrate | Distribution of labeled in product |
|-------------------------------|---------------------------------------------|-----------------------------------|
|                               | Position 1 | Position 2 | Position 1 | Position 2 |
| Phosphatidylglycerol (from A. agilis) | 16:1       | 18:1       | %          | % label    |
| Phosphatidylinositol (from yeast) | 15         | 17         | 36         | 58         |

* cis-Vaccenic acid (27).
+ Oleic acid (20).

acly chains and phospholipase A2 treatment in the manner of Hildebrand and Law (15). Table V summarizes the results of this experiment.

**DISCUSSION**

The transalkylenating enzyme in *M. phlei* extracts which catalyzed the formation of derivatives of 10-methylene stearic acid was obtained in soluble form when cells were broken by sonic oscillation. The fact that some activity remained in the membrane particles sedimenting at 100,000 × g may indicate that the enzyme originally resided in the membrane, where its lipid substrate doubtless was localized. The enzyme catalyzed the reaction between S-adenosyl-L-methionine and olefinic acid chains in phospholipid molecules. While it was readily established that S-adenosylmethionine was the more effective methylase donor than methionine, serine, or formate, the nature of the fatty acid substrate proved more difficult to establish. The major problem was that crude extracts contained adequate lipid substrate and were not stimulated by the addition of exogenous lipids. Acetone treatment, while it resulted in some loss of activity, removed a significant, although variable, amount of endogenous lipid substrate. The best preparations showed almost 100-fold stimulations when exogenous phospholipids were added, although 10-fold stimulations were much more common. Free oleic acid gave no stimulation of synthesis of 10-methylene stearate derivatives.

Added phospholipids were alkylenated on the fatty acid chains, and the otherwise unaltered phospholipids could be extracted and recovered by thin layer chromatography. Hydrolysis and gas-liquid chromatography showed that the C16 and C18 olefinic acid chains were alkylenated to give the C17 and C19 acid derivatives. The ratio of products reflected to some degree the composition of the substrate phospholipids. Experiments designed to determine whether the alkylenating enzyme had a preference for olefinic acid at position 1 or 2 of the glyceride gave perplexing results. Natural phospholipids of different fatty acid compositions were used as substrates. The alkylenated chains were labeled from S-adenosylmethionine-methyl-14C, and product acids from each position were isolated after treatment with phospholipase A2, which specifically hydrolyzes an acid esterified at position 2 of a phosphatide. With phosphatidylyglycerol and phosphatidylinositol as substrates, the enzyme alkylenated predominantly position 2 of the inositide, but preferred position 1 of the phosphatidylyglycerol by a factor of 2:1. An important difference between these substrates was the absence of oleic acid in the phosphatidylyglycerol. This bacterial phospholipid contained only palmitoleic and cis-vaccenic acids (27). Virtually no 19-carbon alkylenated acids were made from cis-vaccenic acid (Table III). However, appropriate olefinic precursor acids were present at both positions 1 and 2 of both substrates, yet the alkylation position differed for the two. A definitive solution to the problem of what determines positional specificity for the alkylenating enzyme must await the availability of appropriate phospholipid substrates with two identical olefinic chains.

It is of interest to compare the properties of the *Mycobacterium* alkylenating enzyme with the cyclopropane synthetase enzymes from *Clostridium* and *Serratia* (10, 14). All three of these enzymes catalyze the alkylenation of olefinic acid chains in phospholipid molecules, with S-adenosylmethionine as a methylene donor. The products are similar, except that the *Mycobacterium* enzyme gives a branched methylene group instead of a cyclopropane ring. All three enzymes are soluble when the cells are broken by sonic disruption or similar drastic treatments. The *Mycobacterium* and *Serratia* enzymes contain adequate amounts of endogenous phospholipid substrate (14). The *Clostridium* enzyme shows a preference for phosphatidylethanolamine and for the fatty acid chain in position 1 (15, 28). Phosphatidylyglycerol and phosphatidylinositol are better substrates for the *Mycobacterium* enzyme and the positional specificity is less well defined. Both *Mycobacterium* and *Clostridium* show an unusual distribution of fatty acids in the phosphatides, in which unsaturated and branched or cyclopropane acids predominate in position 1 (15, 29).

Cyclopropane synthetase from *Clostridium* is stimulated several-fold when the phosphatidylethanolamine substrate is mixed with an anionic detergent. The *Mycobacterium* alkylating enzyme is not stimulated appreciably by any of several types of detergent.

As in the case of cyclopropane fatty acid synthesis, tuberculostearic acid increases in the stationary phase of growth (3). It seems likely that both processes involve enzymes which alkylenate the membrane phospholipids after these have been deposited by the phospholipid synthetases. They are the only two presently known processes in which fatty acid alkyl chains of phospholipids undergo enzymatic alteration. The definitive raison d’être for such enzymatic processes has yet to be offered.

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*J. Biol. Chem.* 1970, 245:701-708.

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