Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maddy, A. H. (1966), *Biochim. Biophys. Acta 117*, 193.
Marchesi, V. T., and Steers, E. (1968), *Science 159*, 203.
Marchesi, V. T., Steers, E., Tillack, T. W., and Marchesi, S. L. (1969), *in* The Red Cell Membrane, Structure and Function, Jamieson, G. A., and Greenwald, T. J., Ed., Philadelphia, Pa., Lippincott, p 117
Mazia, D., and Ruby, A. (1968), *Proc. Natl. Acad. Sci. 61*, 1005.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Rega, A. F., Weed, R. I., Reed, C. F., Berg, G. C., and Rothstein, A. (1967), *Biochim. Biophys. Acta 147*, 297.
Rosenberg, S. A., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1985.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Schneiderman, L. J., and Junga, I. G. (1968), *Biochemistry 7*, 2281.
Shapiro, A. L., Vinuela, E., and Maizell, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Tanford, C. (1961), *in* Physical Chemistry of Macromolecules, New York, N. Y., Wiley.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Am. Chem. Soc. 89*, 729.
Tillack, T. W., Marchesi, S. L., Marchesi, V. T., and Steers, E. (1970), *Biochim. Biophys. Acta* (in press).
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zwaal, R. F. A., and Van Deenan, L. L. M. (1968), *Biochim. Biophys. Acta 163*, 44.

The Synthesis of Long-Chain Fatty Acids by a Cell-Free System from *Mycoplasma laidlawii* A*

Shlomo Rottem and Charles Panos

ABSTRACT: A soluble system from *Mycoplasma laidlawii* A for the synthesis of long-chain saturated fatty acids from acetate is described. In addition to partial requirements for adenosine triphosphate, $MgCl_2$, and reduced nicotinamide–adenine dinucleotide phosphate, this system exhibited an absolute need for malonyl coenzyme A. This soluble system contained a high level of acyl carrier protein activity but a very low acetyl carboxylase activity. Highest fatty acid synthetase and "acyl carrier protein" activities were found in cells harvested during the stationary phase of growth. The products of this synthetase system were only saturated fatty acids, mainly stearic (83.2%). Palmitic (6.6%) and arachidic (10.1%) acids were also formed. Likewise, only saturated fatty acids were formed when β-hydroxydecanoic acid replaced acetate in this synthetase system. The addition of a β-hydroxy thioester dehydrase preparation from *Escherichia coli* to this mycoplasma-soluble system resulted in the formation of both saturated and unsaturated acids. Of the unsaturated fatty acids formed, hexadecenoic acid predominated.

Growing cells and washed suspensions of the nonsterol-requiring *Mycoplasma laidlawii* are capable of incorporating [^{14}C]acetate into long-chain saturated fatty acids (Rottem and Razin, 1967a; Pollack and Tourtellotte, 1967). However, the inability to detect any radioactivity in its unsaturated fatty acid content was supported by nutritional experiments showing a need for an octadecenoic acid for growth of this organism (Razin and Rottem, 1963; Razin *et al.*, 1966). Recent elongation studies have shown that while a closely related mycoplasma, *Mycoplasma* sp. KHS, was able to form octadecenoic acids from short-chain monoenoic acid precursors (Panos and Henrikson, 1969), *M. laidawii* A elongated such precursors mainly to hexadecenoic acids (Panos and Rottem, 1970).

These studies represent the first report of information concerning the *de novo* synthesis of saturated and unsaturated fatty acids by a mycoplasmal cell-free system. These results have been presented in preliminary form (Rottem and Panos, 1969).

Experimental Section

Organism and Growth Conditions. Mycoplasma laidlawii (oral strain) was obtained from S. Razin (The Hebrew University, Hadassah Medical School, Jerusalem, Israel) and is related to *Mycoplasma laidlawii* A (Rottem and Razin, 1967b). Cells were grown in 1–3 l. of a modified Edward medium (Razin, 1963) containing 2% PPLO serum fraction (Difco Laboratories, Detroit, Mich.). Growth was estimated by turbidity at 540 mμ. Cells were harvested after 22–30-hr

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incubation at 37° by centrifugation (17,000*g* for 20 min) and washed once with 0.25 M NaCl containing 0.01 M 2-mercaptoethanol. Since a constant relationship between turpidity and colony forming units (cfu) does not exist beyond the logarithmic phase of growth, the latter method was employed for the estimation of growth when stationary phase cells (Table II) were examined.

Preparation of Cell-Free Extract. Washed sedimented cells were resuspended in an ice-cold solution of 0.01 M 2-mercaptoethanol and broken by alternately freezing and thawing (four times) in an alcohol–Dry Ice bath. Unbroken cells and membranes were removed by centrifugation (34,000*g* for 45 min), $MgCl_2$ (final concentration of 0.02 M) was added, and the supernatant was centrifuged at 100,000*g* for 2 hr. After removal of the sediment, such cell-free extracts were used for assessing fatty acid synthetase activity.

Assays. Fatty acid synthesis was determined by incubating aliquots of this cell-free extract (2 mg protein) at 37° for 30 min with ^{14}C-labeled compounds and measuring the amount of radioactivity incorporated into long-chain fatty acids. A standard reaction mixture contained (in μmoles): triethanolamine-HCl buffer (pH 7.4) 30, 2-mercaptoethanol 5, $MgCl_2$ 5, ATP 1, malonyl-CoA 0.2, NADPH 1, sodium acetate 0.5, and 0.25, μCi of either 1-[^{14}C]sodium acetate (40 mCi/mmole) or 2-[^{14}C]sodium acetate (55.3 mCi/mmole) in a total volume of 1 ml. Elongation of β-hydroxydecanoic acid was tested in a similar reaction mixture with 0.25 μmole of β-hydroxydecanoic acid and 2-[^{14}C]malonyl-CoA (0.25 μmole, 150,000 cpm) replacing sodium acetate and [^{14}C]-acetate. All reactions were stopped with 0.4 ml of 50% (w/v) KOH, saponified in a boiling-water bath for 30 min, acidified, and the long-chain fatty acids were extracted twice with 5-ml portions of petroleum ether (bp 37.5–46.8°). The organic phase was washed with water, transferred to scintillation vials, evaporated to dryness under nitrogen, and 10 ml of scintillation fluid [3 g of 2,5-diphenyloxazol and 0.1 g of 1,4-bis-(5-phenyloxazolyl-2)-benzene in 1 l. of toluene] was added. Samples were counted in a Packard TriCarb scintillation counter. Acetyl-CoA carboxylase was assayed by the method of Waite and Wakil (1962) using [^{14}C]$NaHCO_3$ (55.3 mCi/mmole) and acetyl-CoA that was synthesized from acetic anhydride according to Simon and Shemin (1953). One unit of enzyme is defined as that which catalyzed the formation of 1 μmole of malonyl-CoA/min. Acyl carrier protein content was determined by the malonylpantetheine–CO_2 exchange reaction (Majerus *et al.*, 1964). This reaction mixture, in a total volume of 0.25 ml, contained (in micromoles): imidazole-HCl buffer (pH 6.2) 25, 2-mercaptoethano 110, caproylpantetheine 0.1, malonyl-CoA 0.2, [^{14}C]$NaHCO_3$ (0.08 mCi/mmole) 6.25, 500 μg of cell-free extract protein, and an excess of enzyme fraction A prepared from *Escherichia coli* B (Alberts *et al.*, 1963). Acyl carrier protein from *E. coli* was prepared according to Majerus *et al.* (1964) and served as a control. Both acyl carrier protein from *M. laidlawii* and *E. coli* were incubated for 15 min at 37° in the presence of 0.025 M 2-mercaptoethanol before addition to reaction mixtures to assure reduction of all sulfhydryl groups. After 15-min incubation at 30° the reaction was stopped by addition of 0.05 ml of cold perchloric acid (10% w/v). Aliquots were then transferred to scintillation vials and heated (50°) for 12 hr. After addition of 2 ml of NCS solubilizer (Amersham/Searle Corp., Des Plaines, Ill.) and 10 ml of the toluene scintillation liquid described above the vials were counted. Results are expressed as mμmoles of CO_2 fixed per mg of cell-free protein per 1 min or μmoles of CO_2/mg of protein per 15 min.

Analytical Methods. Protein in cell fractions was determined according to Lowry *et al.* (1951). Decarboxylation of fatty acids was carried out in Thunberg tubes as detailed by Goldfine and Bloch (1961) modified by the use of NCS solubilizer in the side arm for trapping the liberated CO_2. Separation of saturated, unsaturated, and hydroxy fatty acid methyl esters was achieved by thin-layer chromatography on silica gel G plates containing 15% (w/v) $AgNO_3$ and 0.01% (w/v) Rhodamine 6G. The developing solvent system was petroleum ether (bp 38–46°): diethyl ether (9:1, v/v) and the acids were visualized by ultraviolet light. A gas chromatograph (Model 700, F & M Corp., Avondale, Pa.) equipped with a polar column (12 ft × 0.5 in., 10% w/w of butane diol succinate or diethylene glycol succinate on HMDS-treated Chromosorb W, 80–100 mesh) was used for the identification of the products of fatty acid synthesis. This chromatograph was equipped with a thermistor detector and a 1-mV range (Leeds and Northrup, Philadelphia, Pa.) recorder. Newly synthesized radioactive fatty acids were extracted from reaction mixtures as described above, methylated and mixed with standard mixtures of saturated or unsaturated fatty acids, resolved, and collected directly into scintillation vials containing scintillation fluid (10 ml) for counting. For fatty acid composition of whole cells, *M. laidlawii* A was grown and harvested and the fatty acids were extracted as described before (Henrikson and Panos, 1969). Esterified fatty acids were analyzed by capillary gas chromatography (Panos *et al.*, 1966) using a polar column (150 ft × 0.01 in.) coated with Carbowax K-20M+V-93 (99:1).

Reagents. Capric, lauric, myristic, palmitic, stearic, arachidic, *cis*-5-tetradecenoic, myristoleic, palmitoleic, and *cis*-vaccenic acids were purchased from the Hormel Institute, Austin, Minn. Lauroleic acid was the product of Applied Science Laboratories, State College, Pa. All were greater than 99% pure. β-Hydroxydecanoic acid was isolated according to Jarvis and Johnson (1949) from *Pseudomonas aeruginosa* rhamnolipid obtained from J. A. Hayashi (Presbyterian St. Lukes Hospital, Chicago, Ill.). 1-[^{14}C]Sodium acetate (40 mCi/mmole) and 2-[^{14}C]sodium acetate (55.3 mCi/mmole) were the products of Volk Radiochemicals, Burbank, Calif. CoA and malonyl-CoA were purchased from P-L Biochemicals, Milwaukee, Wis. 2-[^{14}C]Malonyl-CoA was synthesized from 2-[^{14}C]malonic acid (8.73 μCi/mmole, New England Nuclear Corp., Boston, Mass.) according to Trams and Brady (1960). Caproylpantetheine was synthesized as detailed by Simon and Shemin (1953). β-Hydroxydecanoyl thioester dehydrase was isolated from *E. coli* B as described by Norris *et al.* (1964).

Results

A system for the synthesis of long-chain fatty acids from acetate by a nonparticulate preparation from *M. laidlawii* A is described. This system is completely soluble and could not be sedimented even after centrifugation at 100,000*g* for 2 hr. Although soluble preparations from this mycoplasma required NADPH, $MgCl_2$, ATP, and malonyl-CoA for maximal enzymic activity, complete cessation of long-chain fatty acid synthesis occurred only when malonyl-CoA was

TABLE I: Incorporation of 1-[^{14}C]Acetate into Fatty Acids by a Cell-Free System from *M. laidlawii* A.

Reaction Mixture	Radioactivity (cpm/mg of protein)	
	Total Fatty Acids	Carboxyl Group[a]
Complete[b]	1454	12
Less malonyl-CoA	36	4
Less ATP	1062	10
Less $MgCl_2$	620	0
Less NADPH	278	6
Less NADPH but added NADH	740	0

[a] By measurement of CO_2 liberated from newly synthesized fatty acids after decarboxylation; [b] Reaction mixture (in μmoles): TEA-HCl buffer (pH 7.4), 30; $MgCl_2$, 5; 2-mercaptoethanol, 5; ATP, 1; NADPH, 0.5; malonyl-CoA, 0.2; sodium acetate, 0.5; 0.2 μCi of 1-[^{14}C]acetate (54.7 mCi/mM) and 2 mg of cell protein. Total volume = 1 ml. Sodium bicarbonate (5 μmoles) was added when malonyl-CoA was omitted.

omitted (Table I). Likewise, ^{14}C derived from isotopic acetate could not be detected in the carboxyl group of newly synthesized fatty acids even in the presence of bicarbonate, indicating that none of the acetate is converted into malonyl-CoA. A direct assay of acetyl-CoA carboxylase revealed that the activity of this enzyme in such cell-free preparations was very low (2.76 μunits/mg of protein). No acetyl-CoA carboxylase activity was ever detected in membrane or ribosomal preparations from this organism.

The optimal temperature for fatty acid synthetase activity in this system was 37°. Practically no activity occurred below 15° and the Q_{10} calculated between 27 and 37° was 2.55. Also, no sharp pH optimum was noted and enzymic activity remained more or less constant over a pH range of 7.0–7.8. Enzymic activity declined sharply below pH 7.0 and above pH 7.8.

Fatty acid synthetase activity was highest in cells harvested at the stationary phase of growth (Table II). No differences were noted between early logarithmic and stationary phase cells with respect to optimal conditions and cofactor requirements for activity. The cell-free extract from *M. laidlawii* A was found to be highly active in the malonylpantetheine–CO_2 exchange reaction indicating the presence of a protein which acts similar to the acyl carrier protein of the fatty acid synthetase system from *E. coli* (Alberts *et al.*, 1963). This exchange reaction did not proceed when either membranes or sediment, obtained during the preparation of mycoplasmal cell-free extracts, were used. As is also apparent in Table II, marked changes in the synthetase activity of cell-free extracts, from cells harvested at various phases of growth, were not accompanied by proportional changes in their "acyl carrier protein" content. The specific activity of acyl carrier protein from mycoplasmal extracts ranged from 5.6 to 17.2 (mμmoles of [^{14}C]CO_2 fixed/mg of protein per min) as compared with 5.7 in a crude suspension from *E. coli* described by Majerus *et al.* (1964).

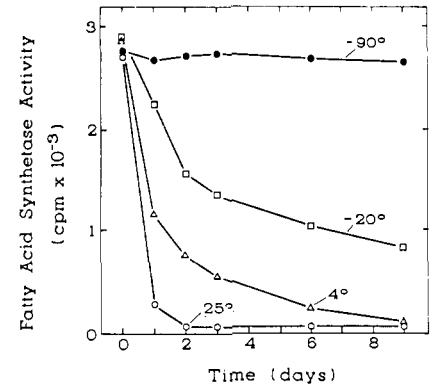

FIGURE 1: Stability of fatty acid synthetase activity from *M. laidlawii* A to prolonged incubation at various temperatures. Activity determined by incorporation of 2-[^{14}C]acetate into long-chain saturated fatty acids.

The fatty acid synthetase activity of cell-free extracts was found to be extremely sensitive to storage (Figure 1). Over a period of 3 weeks, no appreciable decrease in enzymic activity occurred at −90°. Higher temperatures were accompanied by quick inactivation, preventing further purification attempts. Heating of cell-free extracts also resulted in quick inactivation of all synthetase activity. The acyl carrier protein from this mycoplasma, however, was found to be much more stable (Figure 2). Over 50° remained active in the CO_2 exchange reaction even after heating for 5 min at 90°.

Figure 3 illustrates the effect of both coenzyme A and an acyl carrier protein preparation from *E. coli* on the fatty

TABLE II: Fatty Acid Synthetase and Acyl Carrier Protein Activities in *M. laidlawii* A Cells Harvested at Various Growth Phases.

Age of Culture (hr)	Cfu[a]/ml	Fatty Acid Synthetase Activity[b] (cpm/mg of protein)	Acyl Carrier Protein Activity[c] (mμmoles of CO_2/mg of protein per min)
17	5.2×10^9	160	10.6
21	6.3×10^{10}	1000	14.2
25	6.2×10^{10}	1998	17.0
29	5.4×10^{10}	1640	17.2
42	3.5×10^{10}	1241	5.6

[a] Cfu = colony forming units. [b] Reaction mixture (in μmoles): TEA–HCl buffer (pH 7.4), 30; $MgCl_2$, 5; 2-mercaptoethanol, 5; ATP, 1; NADPH, 0.5; malonyl-CoA, 0.2; sodium acetate, 0.5; 0.2 μCi of 1-[^{14}C]acetate (54.7 mCi/mM) and 2 mg of cell protein. Total volume = 1 ml. [c] Reaction mixture (in μmoles): imidazole-HCl buffer (pH 6.2) 25, 2-mercaptoethanol 10, caproylpantetheine 0.1, malonyl-CoA 0.2, [^{14}C]-$NaHCO_3$ (0.08 mCi/mmole) 6.25, 600 μg of cell-free extract protein, and an excess of enzyme fraction A prepared from *E. coli* B. Total volume = 0.25 ml.

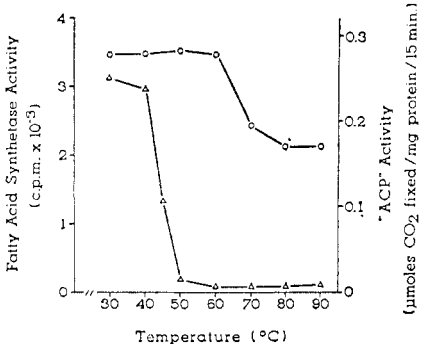

FIGURE 2: Temperature sensitivity of the fatty acid synthetase and acyl carrier protein activities from *M. laidlawii* A. synthetase (Δ) and acyl carrier protein (○) activities assayed by 2-[^{14}C]acetate incorporation into long-chain fatty acids after enzyme aliquots were heated for 5 min at temperatures indicated.

acid synthetase activity of *M. laidlawii* A. A parallel inhibition of activity was obtained with both substances. No inhibition was observed when myristic, palmitic, stearic, oleic, linoleic, or linolenic acids were added to reaction mixtures in concentrations ranging from 2 to 50 μg per ml. In some experiments higher concentrations (25–50 μg/ml) of unsaturated fatty acids were stimulatory, increasing fatty acid synthetase activity up to 70%.

The composition of fatty acids synthesized by soluble cell-free extracts was determined by thin-layer chromatography of the extracted, methylated fatty acid mixture followed by packed-column chromatography. The efficiency of collecting from packed columns coated with butane diol succinate was 67–72% as judged by the recovery of labeled fatty acid standards. The composition of such newly synthesized fatty acid mixtures were comprised of only saturated acids of which stearic acid predominated (Figure 4). The relative composition of the saturated fatty acids newly synthesized by the soluble cell-free system and those extracted from intact cells of *M. laidlawii* A grown in a lipid-preextracted medium is shown in Table III. Most notable is the low amount of

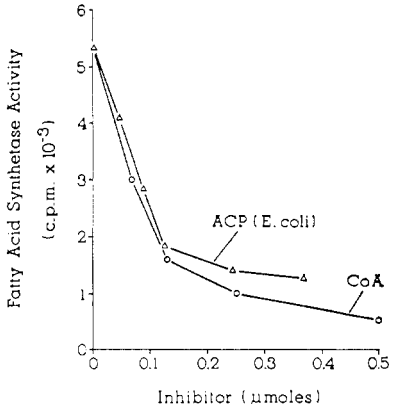

FIGURE 3: Inhibition of mycoplasmal fatty acid synthetase activity by coenzyme A and acyl carrier protein from *E. coli*. Inhibitors added individually to reaction mixtures at zero time and inhibition determined by decrease in 2-[^{14}C]acetate incorporation into long-chain saturated fatty acids.

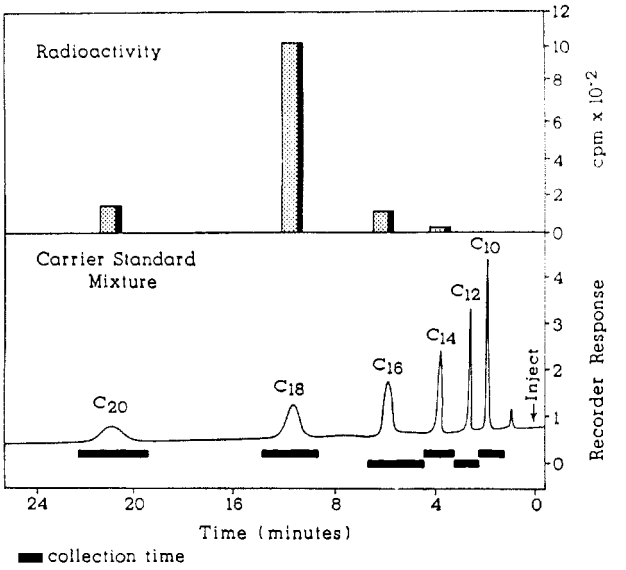

FIGURE 4: *De novo* synthesis of long-chain saturated fatty acids by a cell-free system from *M. laidlawii* A. Distribution of radioactivity in fatty acids after addition of 2-[^{14}C]acetate to a cell-free system. Fractions collected by packed-column (diethylene glycol succinate) gas chromatography.

myristic acid and the high concentration of stearic acid among the products of the cell-free system as compared with their content in intact cells.

In addition to the incorporation of acetate into newly synthesized long-chain fatty acids, this cell-free system was capable of elongating the β-hydroxydecanoic acid isolated from *Pseudomonas aeruginosa* rhamnolipid. Figure 5 illustrates that by using 2-[^{14}C]malonyl-CoA in the reaction mixture, labeling could be detected only in the saturated acid fraction, indicating that a β-hydroxy compound is an intermediate in the biosynthesis of saturated fatty acids. The cell-free extracts in these experiments had to be passed through a column (0.2 × 1 in.) of activated charcoal in 0.05 M triethanolamine-HCl buffer (pH 7.4) and eluted with this same buffer. Without charcoal treatment, cell-free extracts continued to incorporate 2-[^{14}C]malonyl-CoA even in the absence of β-hydroxydecanoic acid, presumably because

TABLE III: Composition of Saturated Fatty Acids Synthesized by a Cell-Free System and Intact Cells of *M. laidlawii* A.

Fatty Acids	Per cent of Total Saturated Fatty Acids[a]	
	Cell-Free System	Whole Cells
Lauric	0	15.1
Myristic	0.1	45.8
Palmitic	6.6	30.0
Stearic	83.2	9.0
Arachidic	10.1	0

[a] Fatty acids determined by gas chromatography.

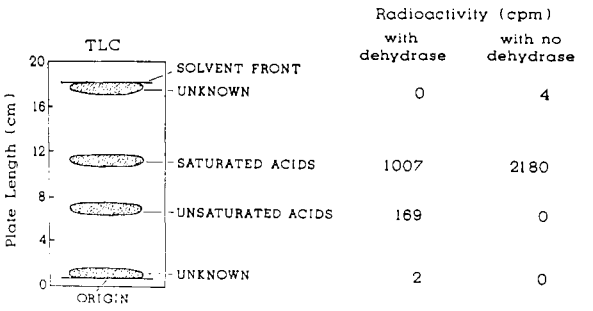

FIGURE 5: Thin-layer chromatography for the isolation of newly synthesized long-chain fatty acids from 2-[^{14}C]acetate by a cell-free system from *M. laidlawii* A in the presence and absence of β-hydroxydecanoate thioester dehydrase from *E. coli* B. Thin-layer chromatography plate: silica gel G with 15% $AgNO_3$ and Rhodamine 6G (0.01%). Solvent system: petroleum ether–diethyl ether (9:1, v/v). Visualization by ultraviolet light.

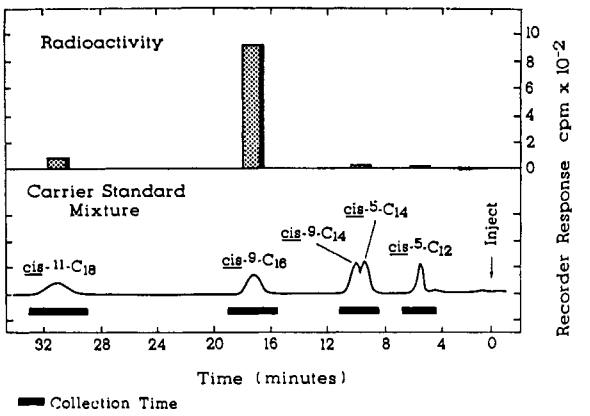

FIGURE 6: *De novo* synthesis of long-chain unsaturated fatty acids by a cell-free system from M. laidlawii A containing β-hydroxy thioester dehydrase from *E. coli*. Distribution of radioactivity in fatty acids after addition of 2-[^{14}C]acetate to the cell-free system. Fractions collected by packed column (butane diol succinate) gas chromatography.

of a relatively high free fatty acid content in such mycoplasmal extracts.

The addition of a β-hydroxydecanoyl thioester dehydrase preparation from *E. coli* B (Kass *et al.*, 1967) to the cell-free system from *M. laidlawii* A resulted in the formation of long-chain unsaturated fatty acids to the extent of 15% of the total acids synthesized (Figure 6). This β-hydroxydecanoyl thioester dehydrase preparation was free of condensing enzymes as judged by the lack of fatty acid synthesis in the absence of the mycoplasmal cell-free extract. Esterified unsaturated and saturated fatty acids from such reaction mixtures were separated by thin-layer chromatography. Unsaturated acid esters were further purified by rechromatography on similar thin-layer plates. Such esterified and labeled monoenoic acid fractions were next mixed with a suitable carrier mixture, resolved by packed-column chromatography and collected for counting as has been detailed above. It should be noted that, surprisingly, the semipreparative packed column used in these studies (butane diol succinate liquid phase) was capable of separating *cis*-9-tetradecenoic acid from *cis*-5-tetradecenoic acid. However, resolution was poor. Figure 6 shows an analysis of the unsaturated long-chain fatty acid content synthesized by cell-free extracts containing the β-hydroxydecanoyl thioester dehydrase preparation from *E. coli* B. A hexadecenoic acid was the predominant component in these preparations while an octadecenoic acid accounted for only 9% of the total unsaturated mixture. This synthesis of only a small amount of an octadecenoic acid is in sharp contrast to the resulting octadecanoic acid content of 83% obtained during only saturated acid formation. The high substrate specificity of the β-hydroxydecanoyl thioester dehydrase preparation from *E. coli* B (Brock *et al.*, 1967) suggest that the hexadecenoic acid formed in these studies is palmitoleic (*cis*-9-hexadecenoic) acid.

Discussion

This soluble enzyme system for the synthesis of long-chain fatty acids from *M. laidlawii* A has general properties similar to other synthetases (Lynen, 1967; Vagelos and Alberts, 1960; Lennarz *et al.*, 1962). Although soluble, its stability at only very low temperatures prevented any purification attempts. The synthesis of long-chain fatty acids in the mycoplasma probably occurs by the malonyl-CoA pathway. However, the strict dependence of a cell-free system from *M. laidlawii* A on an external source of malonyl-CoA may be due to its very low activity of acetyl-CoA carboxylase (2.76 μunits/mg of protein) as compared with high specific activities (50–100 μunits/mg of protein) found in extracts from clostridia (Winder *et al.*, 1964) and animal tissues (Waite and Wakil, 1962). Another indication of the low activity of acetyl-CoA carboxylase, which is the first rate-limiting step in the *de novo* synthesis of fatty acids (Vagelos, 1964), by the mycoplasmal cell-free system was the absence of radioactivity in the carboxyl groups of synthesized fatty acids from reaction mixtures containing 1-[^{14}C]acetate and bicarbonate. However, the possibility of a much greater acetyl-CoA carboxylase activity being present within the intact mycoplasma as well as the relatively short duration time of cell-free experiments as compared with the long generation time of the intact organism may explain the anomalous need for an external source of malonyl-CoA for fatty acid synthesis by this mycoplasmal cell-free system.

The fact that the CoA intermediates of the β-oxidation pathway are not intermediates in the synthesis of fatty acids from malonyl-CoA was pointed out before (Wakil, 1961; Lynen, 1961) and the product of the condensation of acetyl-CoA and malonyl-CoA was, instead, found in many cases to be a heat-stable protein-bound compound (Goldman *et al.*, 1963; Majerus *et al.* 1964). Thus far, acyl carrier protein has been implicated in all systems synthesizing fatty acids *de novo* (Vagelos *et al.*, 1966; Lynen, 1967) and to be active in a CO_2:malonyl-CoA exchange reaction (Alberts *et al.*, 1963). The high activity of cell-free extracts from *M. laidlawii* A in the CO_2:malonyl-CoA exchange reaction indicated the presence of a protein component functionally resembling other acyl carrier proteins. Assuming an identical molecular weight for this mycoplasma acyl carrier protein and that of *E. coli* acyl carrier protein (approximately 10,000, Majerus *et al.*, 1964), the content of acyl carrier protein in cell-free extracts of *M. laidlawii* A ranged from 2.4 to 7.1 μg per mg of

cell-free protein as compared with 2.5 μg in a crude extract of *E. coli*. Changes in acyl carrier protein content of mycoplasmal cells harvested at their various growth phases might be one of the reasons for the difference in their fatty acid synthetase activity. However, the disproportionate changes in synthetase activity and acyl carrier protein content over the growth cycle of the mycoplasma indicates that other factors are affecting the relationship of fatty acid synthetase activity with age. Although other enzymic activities of mycoplasmas are known to be greatest during early logarithmic growth (Razin *et al.*, 1968; Rottem and Razin, 1966), highest fatty acid synthetase activity occurred in extracts from cells harvested at the stationary phase of growth. The reason for this difference of synthetase activity between young and old cells remains obscure.

Replacement of the *E. coli* acyl carrier protein by acyl carrier protein preparations from spinach or avocado in an enzyme system from *E. coli* was reported previously (Simoni *et al.*, 1966). Although these systems were still active, these authors found an alteration in the newly synthesized fatty acid. Acyl carrier protein from *E. coli* was found to be markedly inhibitory for the fatty acid synthetase activity of this mycoplasma. However, mycoplasma acyl carrier protein could replace the *E. coli* acyl carrier protein in the CO_2–malonyl-CoA exchange reaction catalyzed by an enzyme fraction from *E. coli*. The inhibition of the mycoplasma synthetase system by *E. coli* acyl carrier protein as well as by CoA seems to be of a competitive nature. Differences in the amino acid composition of various acyl carrier proteins have been reported (Matsumura and Stumpf, 1968; Willecke *et al.*, 1969; Vanaman *et al.*, 1968). Although portions of the amino acid sequence near the active site of various acyl carrier proteins may be similar (Matsumura and Stumpf, 1968; Vanaman *et al.*, 1968), it is still possible that sequential differences between the acyl carrier proteins of *E. coli* and *M. laidlawii* A are responsible for the inhibition of the mycoplasmal fatty acid synthetase by the acyl carrier protein from *E. coli*.

The relative composition of the fatty acids synthesized by the soluble system differed markedly from those found in intact cells of *M. laidlawii* A (Table III). Most notable and apart from the dominance of stearic acid was the presence of a C_{20} saturated fatty acid. However, no unsaturated fatty acids were formed. The factors controlling the termination of fatty acid synthesis and the chain length of the final product have not been elucidated (Kates, 1966).

Available evidence has shown that in certain bacteria β-hydroxydecanoyl thioester is an intermediate in the synthesis of saturated as well as unsaturated acids. This intermediate, when dehydrated to the α,β-decanoyl thioester, is later reduced to a decanoyl thioester which, in turn, is further elongated to form the saturated fatty acids. However, this intermediate can be dehydrated to the β,γ-decanoyl thioester which results in the elongation to unsaturated fatty acids (Brock *et al.*, 1967). A specific dehydrase has been isolated from *E. coli* that is capable of dehydrating the β-hydroxydecanoyl thioester derivatives of acyl carrier protein, CoA, and *N*-acetylcystamine to a mixture of α,β- and β,γ-decanoyl derivatives (Brock *et al.*, 1967; Kass *et al.*, 1967). The addition of this enzyme to an extract from an unsaturated fatty acid auxotrophic mutant of *E. coli* restored the ability of this mutant to form unsaturated fatty acids (Silbert and Vagelos, 1967). The possibility of shifting the fatty acid synthetase activity of *M. laidlawii* A to the formation of an unsaturated fatty acid by the addition of this β-decanoyl thioester dehydrase from *E. coli* established the fact that this enzyme is serving as a branching enzyme in the synthesis of saturated and unsaturated fatty acids, and may indicate that *M. laidlawii* A is lacking both the aerobic and anaerobic pathway for the formation of unsaturated fatty acids. In contrast to the predominance of an octadecanoic acid when no dehydrase was added, the addition of a dehydrase preparation from *E. coli* resulted in the synthesis of a hexadecenoic acid by this mycoplasma cell-free system. This suggests that the inability of growing cells of this organism to further elongate a hexadecenoic acid (Panos and Rottem, 1970) may be due to the lack of a specific dehydrase.

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References

Alberts, A. W., Goldman, P., and Vagelos, P. R. (1963), *J. Biol. Chem. 238*, 447.
Brock, D. J. H., Kass, L. R., and Bloch, K. (1967), *J. Biol. Chem. 242*, 4432.
Goldfine, H., and Bloch, K. (1961), *J. Biol. Chem. 236*, 256.
Goldman, P., Alberts, A. W., and Vagelos, P. R. (1963), *J. Biol. Chem. 238*, 3579.
Henrikson, C. V., and Panos, C. (1969), *Biochemistry 8*, 646.
Jarvis, F. G., and Johnson, M. J. (1949), *J. Am. Chem. Soc. 71*, 4124.
Kass, L. R., Brock, D. J. H., and Bloch, K. (1967), *J. Biol. Chem. 242*, 4418.
Kates, M. (1966), *Ann. Rev. Microbiol. 20*, 13.
Lennarz, W. J., Light, R. J., and Bloch, K. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 848.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Lynen, F. (1961), *Fed. Proc. 20*, 941.
Lynen, F. (1967), *Biochem. J. 102*, 381.
Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1231.
Matsumura, S. and Stumpf, P. K. (1968), *Arch. Biochem. Biophys. 125*, 932.
Norris, A. T., Matsumura, S., and Bloch, K. (1964), *J. Biol. Chem. 239*, 3653.
Panos, C., Cohen, M., and Fagan, G. (1966), *Biochemistry 5*, 146.
Panos, C., and Henrikson, C. V. (1969), *Biochemistry 8*, 652.
Panos, C., and Rottem, S. (1970), *Biochemistry* (in press).
Pollack, J. D., and Tourtellotte, M. E. (1967), *J. Bacteriol. 93*, 636.
Razin, S. (1963), *J. Gen. Microbiol. 33*, 471.
Razin, S., Gottfried, L., and Rottem, S. (1968), *J. Bacteriol. 95*, 1685.
Razin, S., and Rottem, S. (1963), *J. Gen. Microbiol. 33*, 459.
Razin, S., Tourtellotte, M. E., McElhaney, R. N., and Pollack, J. D. (1966), *J. Bacteriol. 91*, 609.
Rottem, S., and Panos, C. (1969), *Bacteriol. Proc.*, P151.
Rottem, S., and Razin, S. (1966), *J. Bacteriol. 92*, 714.

Rottem, S., and Razin, S. (1967a), *J. Gen. Microbiol. 48*, 53.
Rottem, S., and Razin, S. (1967b), *J. Bacteriol. 94*, 359.
Silbert, D. F., and Vagelos, P. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1579.
Simon, E. J., and Shemin, D. (1953), *J. Am. Chem. Soc. 75*, 2520.
Simoni, R. D., Criddle, R. S., and Stumpf, P. W. (1966), *J. Biol. Chem. 242*, 573.
Trams, E. G., and Brady, R. O. (1960), *J. Am. Chem. Soc. 82*, 2972.
Vagelos, P. R. (1964), *Ann. Rev. Biochem. 33*, 139.
Vagelos, P. R., and Alberts, A. W. (1960), *J. Biol. Chem. 235*, 2786.
Vagelos, P. R., Majerus, P. W., Alberts, A. W., Larabee, A. R., and Ailhaud, G. P. (1966), *Fed. Proc. 25*, 1485.
Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968), *J. Biol. Chem. 243*, 6420.
Waite, M., and Wakil, S. J. (1962), *J. Biol. Chem. 237*, 2750.
Wakil, S. J. (1961), *J. Lipid Res. 2*, 1.
Willecke, K., Ritter, E., and Lynen, F. (1969), *European J. Biochem. 8*, 503.
Winder, F. G., Brennan, P., and Ratledge, C. (1964), *Biochem. J. 93*, 635.

In Vitro Study of the Methylation Pathway of Phosphatidylcholine Synthesis and the Regulation of This Pathway in *Saccharomyces cerevisiae**

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ABSTRACT: A particulate, cell-free preparation has been prepared from *Saccharomyces cerevisiae* capable of incorporating the labeled methyl group of *S*-adenosyl-L-methionine, into phosphatidylcholine, phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and ergosterol. When particles are prepared from yeast grown in the presence of choline, they have a much lower rate of labeling of phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and phosphatidylcholine than particles from yeast grown in the absence of choline.

The two preparations are qualitatively similar. They incorporate radioactivity from [*Me*-^{14}C]*S*-adenosyl-L-methionine into phosphatidylcholine at linear rates. Both preparations reach steady-state levels of the methylation intermediates phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine. The preparation from yeast grown in the absence of choline is more active in the synthesis of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine, reaches a higher steady-state level and reaches the steady-state level more rapidly than does the preparation from yeast grown in the presence of choline. The rate of ergosterol labeling is linear and very similar for the two preparations. The two preparations also exhibit the same response to pH and have the same K_m for *S*-adenosyl-L-methionine while the V_{max}'s for the two preparations differ by a factor of 14. The preparations differ in the endogenous concentration of phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, and phosphatidyl-*N,N*-dimethylethanolamine; however, these differences are not of sufficient magnitude to account for the difference in activity between the preparations. A difference in the amount of enzyme is a likely explanation for the differences observed between the two preparations; this presumably results from the repression of enzyme synthesis in the presence of choline.

The synthesis of phosphatidylcholine (PC)[1] is known to occur *via* a number of metabolic pathways. One pathway, the successive methylation of PE, is the sole mode of PC synthesis demonstrated in bacteria (Kaneshiro and Law, 1964). In mammalian systems both the methylation pathway and the synthesis of PC from CDP–choline and D-α,β-diglyceride have been demonstrated (Bremer *et al.*, 1960; Kennedy and Weiss, 1956). The relative importance of these pathways in PC synthesis has been found to be different in different mammalian organs and between the sexes (Bjornstad and Bremer, 1966). Lombardi *et al.* (1969) observed that the *in vivo* incorporation of [*Me*-^{3}H]AMet was higher in rats fed a choline-deficient diet than in rats fed a choline-supplemented diet.

Saccharomyces cerevisiae is able to synthesize PC by the methylation and CDP–choline pathways (Waechter *et al.*,

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[1] The abbreviations used are: PC, phosphatidylcholine; PDME, phosphatidyl-*N,N*-dimethylethanolamine; PMME, phosphatidyl-*N*-monomethylethanolamine; PE, phosphatidylethanolamine; AMet, *S*-adenosyl-L-methionine; +C, cell-free particulate preparation from cells grown in the presence of choline; −C, cell-free particulate preparation from cells grown in the absence of choline.