The Involvement of Thioesterase Activity in Phospholipid Metabolism

by

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Abstract

Crude soluble preparations obtained from anaerobic, light-grown cells of Rhodopseudomonas sphaeroides have been shown to possess a significant level of acyl-CoA thioesterase activity. This enzyme catalyzes the hydrolysis of long chain fatty acyl thioesters of coenzyme A to produce free fatty acids and release coenzyme A (1). Maximal velocities obtained for thioesterase activity utilizing the coenzyme A derivatives of the saturated fatty acid, palmityl-CoA, and the unsaturated fatty acid, oleoyl-CoA, indicate that the enzyme possesses no preference in relation to the degree of saturation of the substrate. Thioesterase activity monitored in asynchronously dividing cultures indicated that the activity increased proportional to cell growth in cells growing anaerobically at a light intensity of 500 ft-c. Following a shift from high (500 ft-c.) to low (50 ft-c.) intensity light, a marked decrease in thioesterase activity was observed. A similar decrease in thioesterase activity was observed just prior to cell division in cultures synchronized by a light shift procedure.

Acknowledgements

I would like to express my appreciation to Dr. Donald R. Lueking for the guidance, understanding and patience he has demonstrated to me throughout my undergraduate work. I consider it a privilege to have spent the past year under the excellent supervision of this man.

In addition, I would like to thank Tom Campbell for his laboratory instruction and Don Welch for his time and patience during the completion of this study.

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Introduction[1]

In mammalian systems, previous studies have shown that newly formed fatty acids are released from the fatty acid synthetase complex by an enzyme termed thioesterase. This enzyme catalyzes the hydrolysis of the thioester bond joining the fatty acid to the fatty acid synthetase complex. The result of this hydrolysis is the formation of free fatty acids which are then reactivated to their CoA thioester form before subsequently being utilized for complex lipid synthesis. Thus, the mode of fatty acid and phospholipid synthesis in mammalian systems provides a rationale for the occurrence of thioesterase activity in these systems.

In bacterial systems, the intermediates and products of fatty acid synthesis also occur covalently attached to a component of the fatty acid synthetase complex. In this instance, however, the fatty acids are covalently attached to a low molecular weight protein termed acyl carrier protein (ACP) which serves to stabilize the fatty acids as they are being synthesized. This fatty acid-ACP portion of the complex is known to be completely dissociable from the rest of the complex and is capable of directly serving as the acyl donor for subsequent lipid synthesis; prior hydrolysis and reactivation of the fatty acid to its coenzyme A thioester form not being required (1).

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[1] The format followed in this text is that of The Journal of Bacteriology, a publication of the American Society for Microbiology.

Thus, a physiological rationale for thioesterase activity in bacterial systems is not immediately apparent even though this enzyme activity has been shown to be present and highly active in prokaryotic organisms (2).

In bacterial systems, newly synthesized fatty acids are utilized solely for the purpose of membrane phospholipid synthesis. Thus, by monitoring thioesterase activity under conditions of varying demands for cellular phospholipids, it should be possible to tentatively ascribe an *in vivo* function to the bacterial thioesterases.

These studies will be conducted employing the non-sulfur, purple, photosynthetic bacterium, *Rhodopseudomonas sphaeroides*. The ease and predictability with which this organism's membrane content can be experimentally manipulated render it ideal for the proposed studies.

Materials and Methods

Organism, Medium, and Growth Conditions--Rhodopsuedomonas sphaeroides strain M29-5 (leu^-, met^-), derived from strain 2.4.7, was obtained from Samuel Kaplan, University of Illinois. The organism was routinely grown in minimal media (initial pH, 7.0) containing per liter: 4 g of succinic acid, 0.1 g of glutamic acid, 0.04 g of aspartic acid, 0.8 g of $(NH_4)_2SO_4$, and additional inorganic salts as specified by Sistrom (3). Media employed for growth of strain M29-5 was supplemented with 50 µg/ml each, of L-leucine and L-methionine.

Stock cultures were maintained at -20°C in the above medium adjusted to 10% (w/v) glycerol.

Incubations were conducted anaerobically in the light (photoheterotrophically) in completely filled screw-cap vessels or in flat-walled vessels (1 to 5 liters) under an atmosphere of 95% N_2 and 5% CO_2. Cells previously adapted to logarithmic phase growth were the inoculum source for studies with asynchronous cell populations. Inocula for studies with synchronous cell populations were adapted as described below. Incubations were at 30-32°C with continuous saturating illumination (500 ft-c.) provided by a bank of 60-Watt Lumiline lamps (Sylvania). Culture growth was followed turbidimetrically using a Klett-Summerson colorimeter equipped with a No. 66 filter. A culture turbidity of 100 photometer units corresponds to a value of 270 µg cellular dry weight/ml culture.

Synchronization Technique--Populations of synchronously dividing cells were obtained by the light-shift technique of Lueking and Campbell (4). Cultures adapted to logarithmic (asynchronous) growth on minimal medium were adapted to log phase growth in high intensity light (500 ft-c.) and were used as the inoculum source for 2 to 5 liter flat-walled vessels under an atmosphere of 95% N_2 and 5% CO_2. Culture growth was followed using a Klett-Summerson colorimeter until the turbidity of the culture was approximately 35 Klett units. At this point, the bottles were subjected to low intensity light (50 ft-c.) for a period of 3.5 hours and then returned to high intensity light. Culture samples, corrected for uniform cell mass, were then removed at 15 minute intervals and the cells were prepared as described below to obtain soluble protein fraction for the measurement of thioesterase activity.

Light Shift Procedure--Cultures adapted to logarithmic (asynchronous) growth on minimal medium were adapted to log phase growth in high intensity light (500 ft-c.) and were used as the source of inocula for 2 to 5 liter flat-walled vessels under an atmosphere of 95% N_2 and 5% CO_2. Culture growth was followed using a Klett-Summerson colorimeter until the turbidity of the culture was approximately 80 Klett units. At this point, the bottles were shifted to low intensity light (50 ft-c.) and culture samples, corrected for uniform cell mass, were removed at 15 minute intervals and the cells were prepared as described below in order to obtain a soluble protein fraction.

Cell Enumeration--The presence and degree of division synchrony was determined by monitoring total cell number using a Petroff-Hausser counting chamber. Samples (1 ml) of the culture were removed at 15 minute intervals and transferred to a tube containing 1 ml of 5% (w/v) formaldehyde.

Preparation of Cell-free Extracts--Soluble fractions possessing the enzyme thioesterase were prepared according to procedures described previously by Lueking et al (5). Cells in the logarithmic phase of growth (approximately 150 Klett units) were harvested by centrifugation at 10,000 rpm for 15 minutes and washed once in 0.05 M KH_2PO_4 buffer. The washed cells were resuspended in 0.05 M phosphate buffer and disrupted by sonication for ten minutes (40% pulse regimen) with a Branson model W-350 sonicator. The resulting suspension was freed of whole cells and debris by centrifugation at 10,000 rpm for 15 minutes and the crude supernatant suspension was centrifuged at 150,000 x g for one hour. The resulting supernatant (soluble fraction) was removed and utilized as the immediate source of thioesterase activity.

Spectrophotometric Assay for Thioesterase--Palmityl-CoA is hydrolyzed in the presence of thioesterase to form palmitic acid and release coenzyme A. The assay system was based upon the quantitation of the sulfhydryl group of CoA employing the thiol reagent, dithionitrobenzoic acid (6). As described by Barnes and Wakil (6), the release of CoA was measured by monitoring the increase in optical density at 412 nm resulting from the production of the colored anion.

A molar extinction coefficient of 13,600 was employed for calculations (7). A Beckman dual beam spectrophotometer was used for these measurements.

A typical assay contained 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0). 0.012 ml of 2.5 mM palmityl-CoA, 0.01 ml of 10 mM DTNB, and 0.100 ml of protein with water being added to a final volume of 1 ml. The initial velocity of the reaction was taken as a measure of thioesterase activity with an enzyme unit being defined as an absorbance reading of 0.001 OD/min. Specific activity was defined as units of activity/mg of protein. All assays were performed at room temperature.

Analytical Determination--Whole cell protein was determined by the method of Lowry et al. (8) employing bovine serum albumin as standard. Calculations of palmityl-CoA and oleoyl-CoA concentrations were performed at 260 nm assuming a millimolar extinction coefficient of 15.9. A Beckman dual beam spectrophotometer was utilized for these measurements.

Results

Characterization of Acyl-CoA Thioesterase Activity--The soluble fraction (supernatant) obtained following centrifugation at 150,000 x g as described in Materials and Methods was utilized as the immediate source of thioesterase activity for the present study (Fig. 1). By comparing units of activity between crude and soluble preparations containing thioesterase, greater than 90% of the thioesterase activity was found to be retained in the soluble fraction indicating that the enzymatic activity is not membrane associated.

As previously stated, the enzyme thioesterase catalyzes the hydrolysis of thioester linkages of acyl-CoA derivatives thereby producing free fatty acids and CoA (Fig. 2). The sulfhydryl reagent, dithionitrobenzoic acid, reacts spontaneously and quantitatively with the free sulfhydryl groups of CoA to generate a CoA disulfide, thus liberating a highly colored anion that absorbs light maximally at 412 nm.

Employing this assay system, the R. sphaeroides thioesterase was shown to be active on both palmityl and oleoyl thioester derivatives of CoA. Utilizing constant levels of the enzyme substrate, palmityl-CoA, thioesterase activity was found to increase in proportion to the quantity of soluble protein present in the assay system. A linear increase in activity was observed from a concentration of 0.015 to 0.110 mg protein/ml (Fig. 3).

Figure 1 -- Protocol for preparation of soluble fraction possessing thioesterase activity. Details of this preparation are given in "Materials and Methods."

PREPARATION OF CELLS

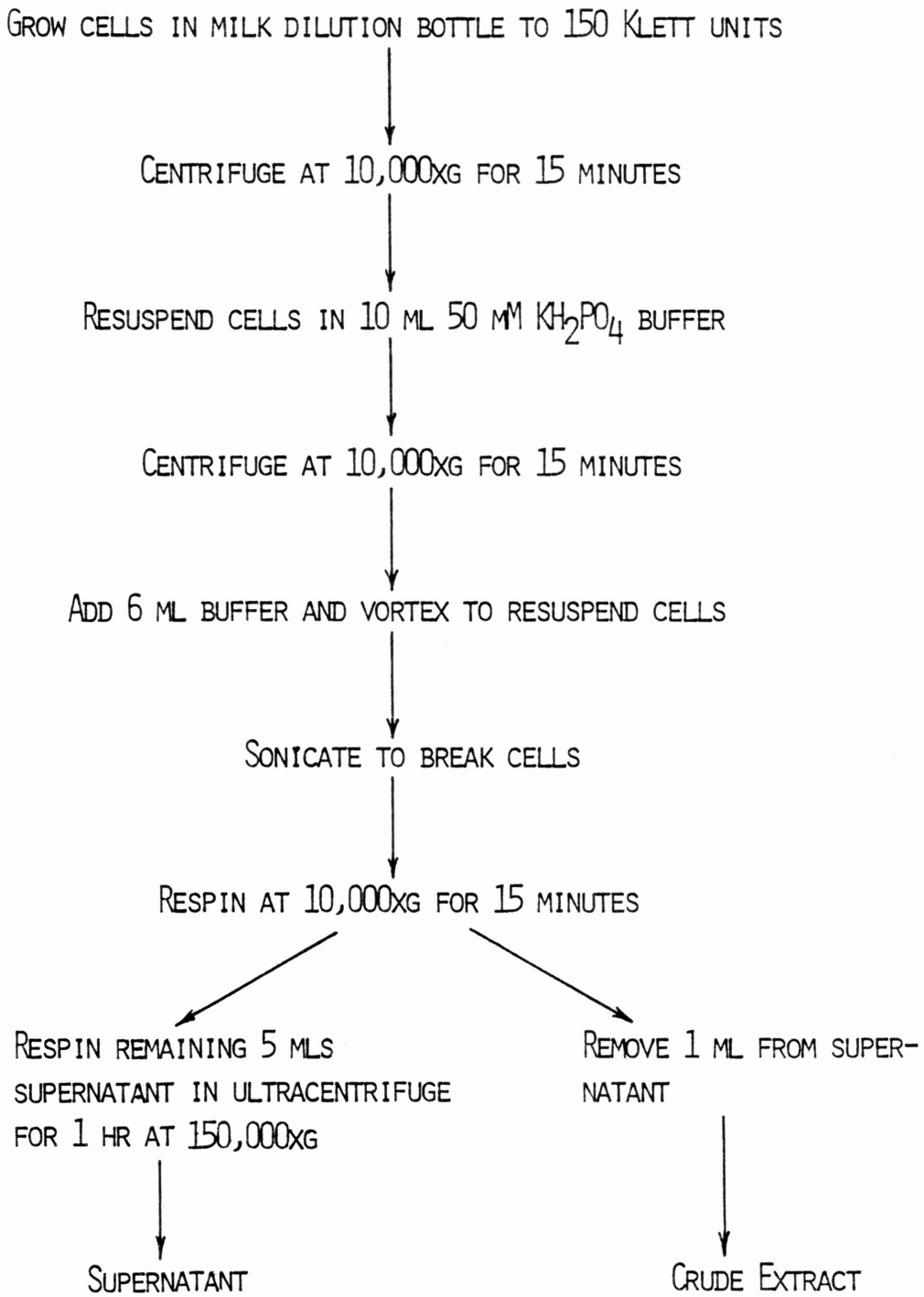


Figure 2 -- Chemistry of the interaction of DTNB with sulfhydryl containing compounds. Details of this assay are described under "Materials and Methods."

PALMITOYL-CoA —THIOESTERASE→ PALMITIC ACID + CoASH

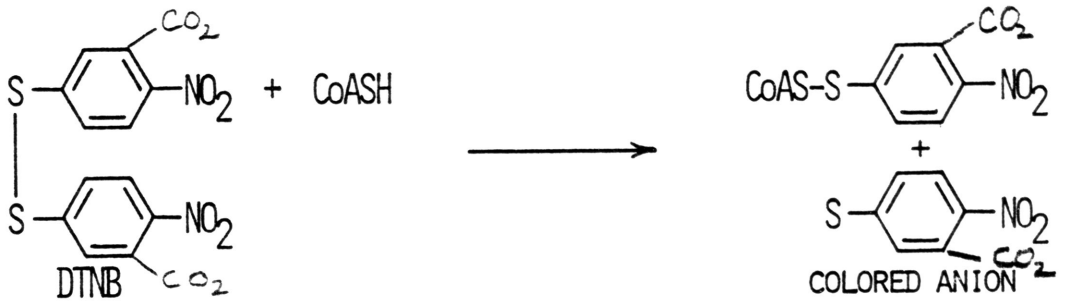
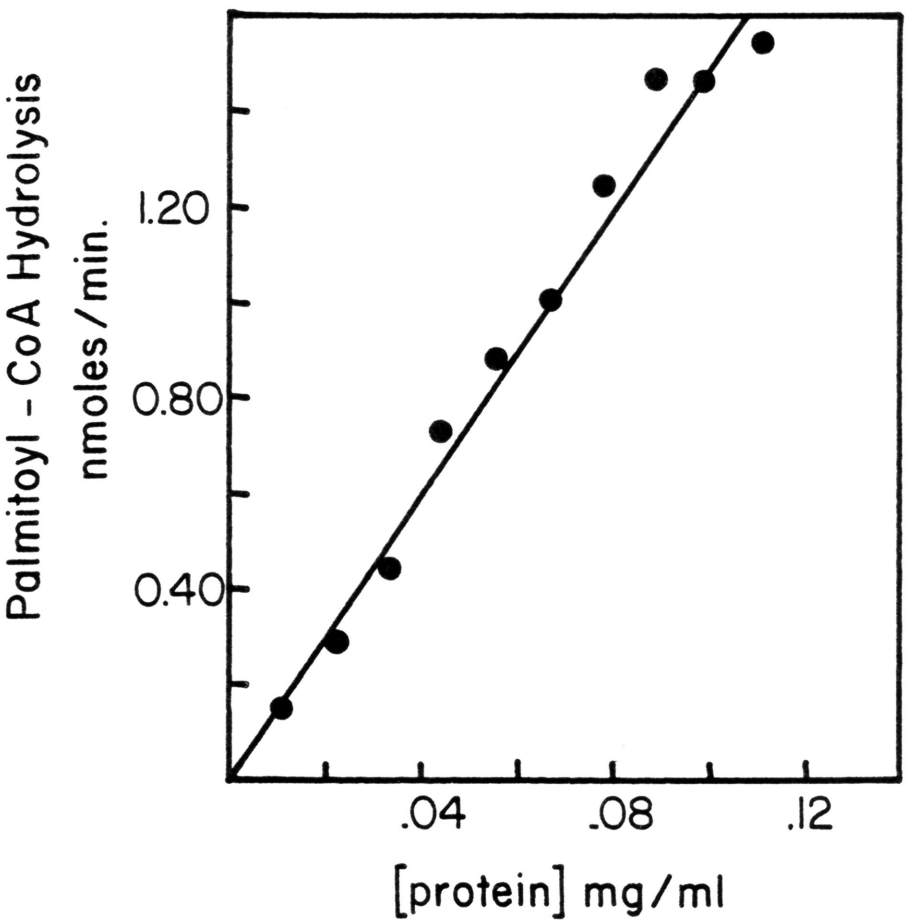


Figure 3 -- Protein dependence of thioesterase activity from photosynthetically grown cells of R. sphaeroides. Components of the assay mixture are given in "Materials and Methods."


Palmitoyl - CoA Hydrolysis
nmoles / min.
1.20
0.80
0.40
.04
.08
.12
[protein] mg / ml

Utilizing thioester derivatives of palmityl-CoA as enzyme substrates, this study showed that a concentration of 12 uM palmityl-CoA supported a maximal level of thioesterase activity. The specific activity obtained for the hydrolysis of palmityl-CoA by the thioesterase was 16.6 nmoles/min/mg protein (Fig. 4).

By replotting the enzyme activity data in the form of a Lineweaver-Burke plot, kinetic constants such as the Vmax and Km were easily determined. Employing palmityl-CoA as the enzyme substrate, a value of 3.3 nmoles/min was obtained for the Vmax and a value of 12.5 uM for the Km (Fig. 5). Similar values were obtained utilizing the thioester derivative of the unsaturated fatty acid, oleoyl-CoA, as the substrate for the reaction. Values for maximal velocity of 2.7 nmoles/min and a Km of 10.0 uM were determined utilizing this latter substrate (Fig. 6).

A comparison of the relative activities of thioesterase on palmityl-CoA and oleoyl-CoA is given in Table 1. The values obtained from this study for these kinetic parameters agree with other values obtained for thioesterases in studies utilizing Escherichia coli (2).

Thioesterase Activity in Asynchronously Dividing Cultures--Having defined the optimal assay conditions, the level of thioesterase activity was monitored in photosynthetically growing cells of R. sphaeroides at a light intensity of 500 ft-c. As shown by Figure 7, thioesterase activity in asynchronously dividing cultures was found to increase in direct proportion to cell growth.

Figure 4 -- Dependence of thioesterase activity on concentrations of acyl-CoA substrates. All incubation mixtures contained .088 mg of soluble protein.

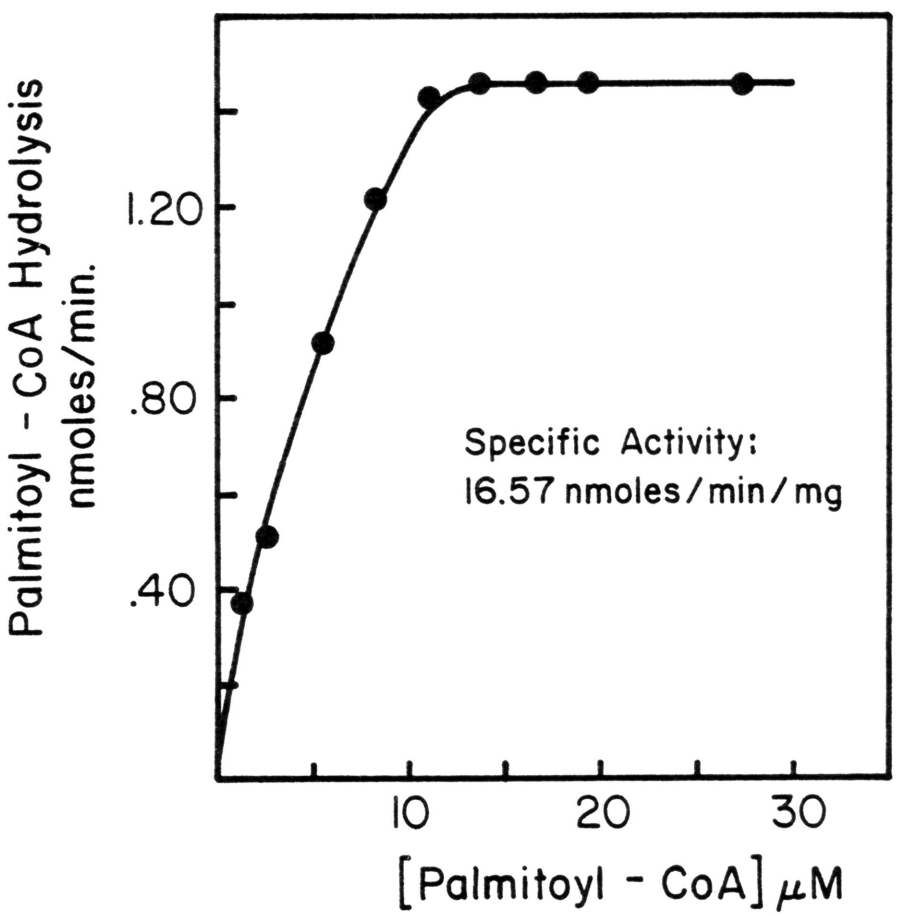

Palmitoyl - CoA Hydrolysis
nmoles/min.
1.20
.80
.40
Specific Activity:
16.57 nmoles/min/mg
10
20
30
[Palmitoyl - CoA] μM

Figure 5 -- Lineweaver-Burke plot of the initial velocity of thioesterase activity utilizing palmityl-CoA as substrate. Values obtained from this plot are given in "Results."

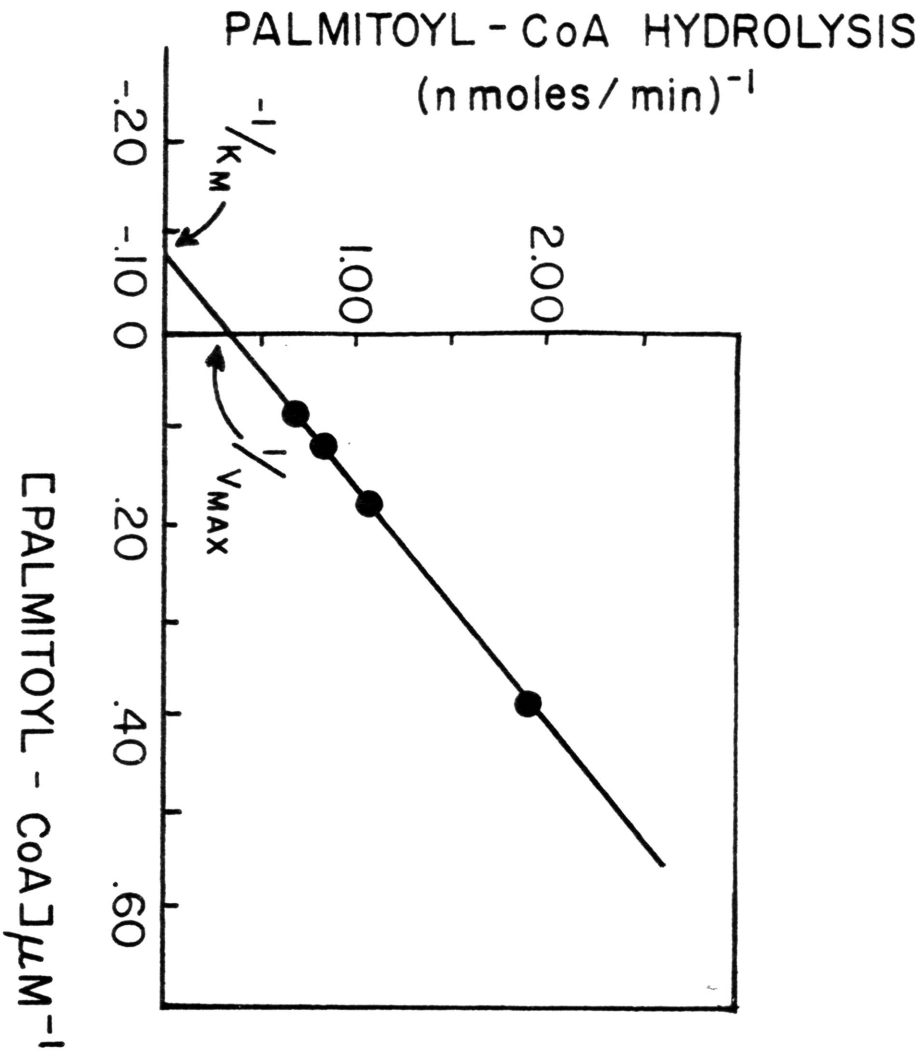

PALMITOYL - CoA HYDROLYSIS
(n moles / min)$^{-1}$
-.20
-.10
0
1.00
2.00
$-1/K_M$
$1/V_{MAX}$
.20
.40
.60
[PALMITOYL - CoA] μM^{-1}

Figure 6 -- Lineweaver-Burke plot of the initial velocity of thioesterase activity utilizing oleoyl-CoA as substrate. Values obtained from this plot are given in "Results."

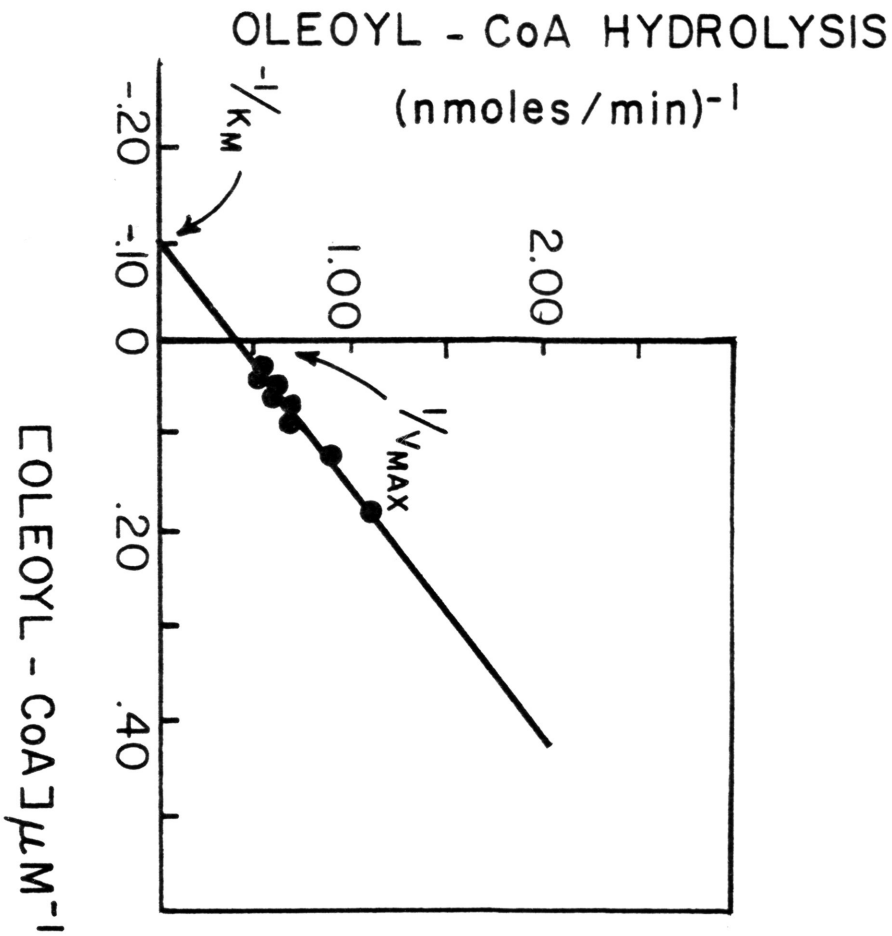
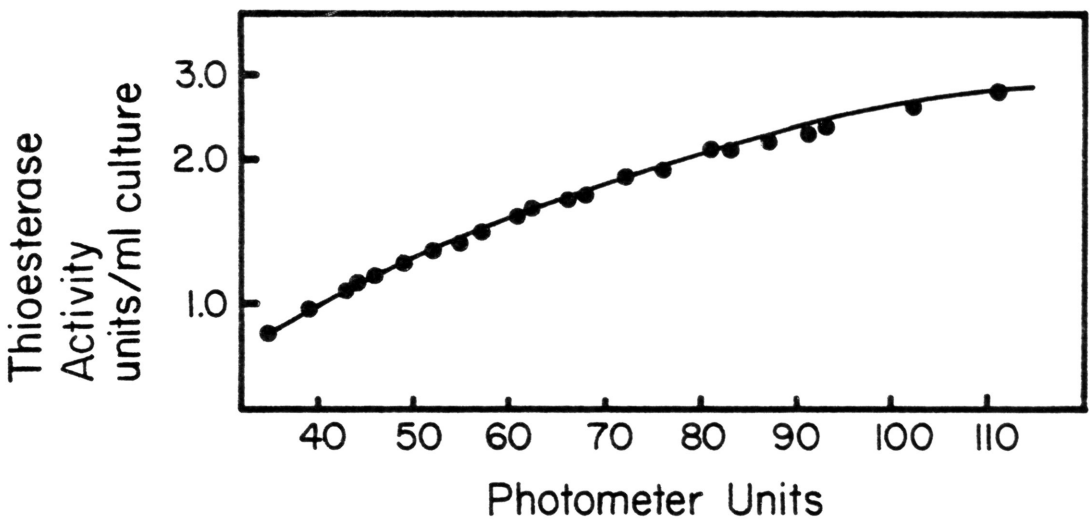

OLEOYL - CoA HYDROLYSIS
(nmoles/min)⁻¹
-1/KM
1/VMAX
-.20
-.10
0
1.00
2.00
.20
.40
[OLEOYL - CoA] μM⁻¹

Table 1 -- A comparison of the relative activities of thioesterase on palmityl-CoA and oleoyl-CoA substrates.

THIOESTERASE ACTIVITY IN PHOTOSYNTHETICALLY GROWN CELLS OF *RHODOPSEUDOMONAS SPHAEROIDES*

Substrate	Km (μM)	Vmax (nmoles/min)	Specific Activity (nmoles/min/mg)
Palmitoyl-CoA	12.5	3.3	16.5
Oleoyl-CoA	10.0	2.6	13.2

Figure 7 -- Thioesterase activity in asynchronously dividing cultures. Thioesterase activity monitored in photosynthetically growing cells of R. sphaeroides at a light intensity of 500 ft-c.


Thioesterase
Activity
units/ml culture
3.0
2.0
1.0
40
50
60
70
80
90
100
110
Photometer Units

Thioesterase Activity During a High to Low Light Transition of High Light Adapted Cells--Having determined the activity of thioesterase in photosynthetically growing cells of R. sphaeroides, control studies of the activity were initiated by monitoring the response of thioesterase activity to an immediate decrease in light intensity (500 to 50 ft-c.). The results of this study showed that enzyme activity increased exponentially until the point of the light shift, at which time, further increases ceased. Enzyme activity then began to increase at a much slower rate than that observed in high intensity light (Fig. 8, a). As predicted, whole cell number experienced a lag phase of approximately 2 hours following the light shift, before cell division occurred (Fig. 8, b). In comparison to the observed increase in cell number, there was a marked decrease in thioesterase activity immediately prior to cell division (Fig. 8, c), suggesting that the enzymatic activity is inhibited by processes occurring at cell division.

Thioesterase Activity in Synchronously Dividing Cultures--In order to determine if the changes in thioesterase activity observed during the light shift corresponded to periods of cell division, thioesterase activity was monitored in synchronously dividing cultures as described in Materials and Methods. In support of the previous observations, thioesterase activity showed a marked decrease in activity immediately prior to cell division (Fig. 9) which has also been previously shown to be the point at which maximum phospholipid synthesis occurs (9). These observations suggest that the modulation of thioesterase activity described is in some way related to the cellular division process.

Figure 8 -- Thioesterase activity during a high to low light transition of high light adapted cells. The light shift procedure is described under "Materials and Methods." a, photometer units; b, total cells per ml of culture; c, thioesterase activity units per ml of culture.

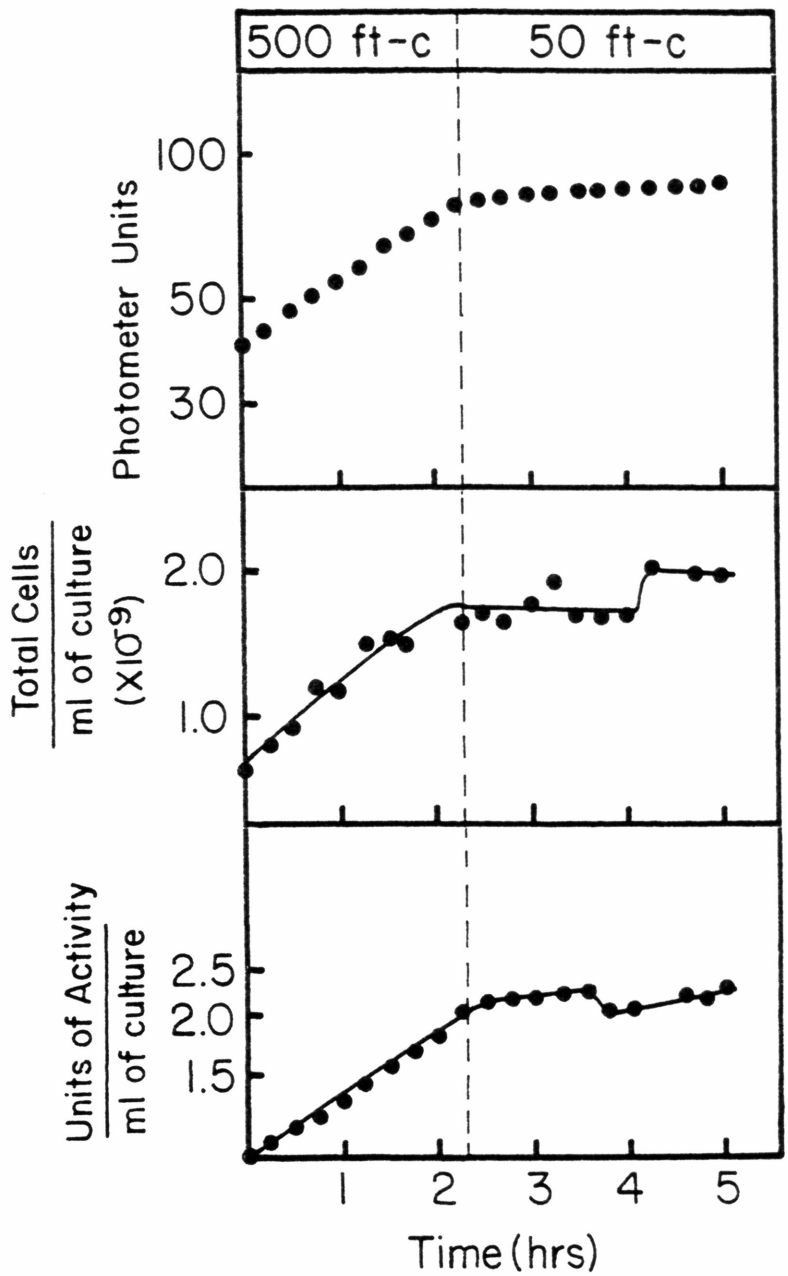
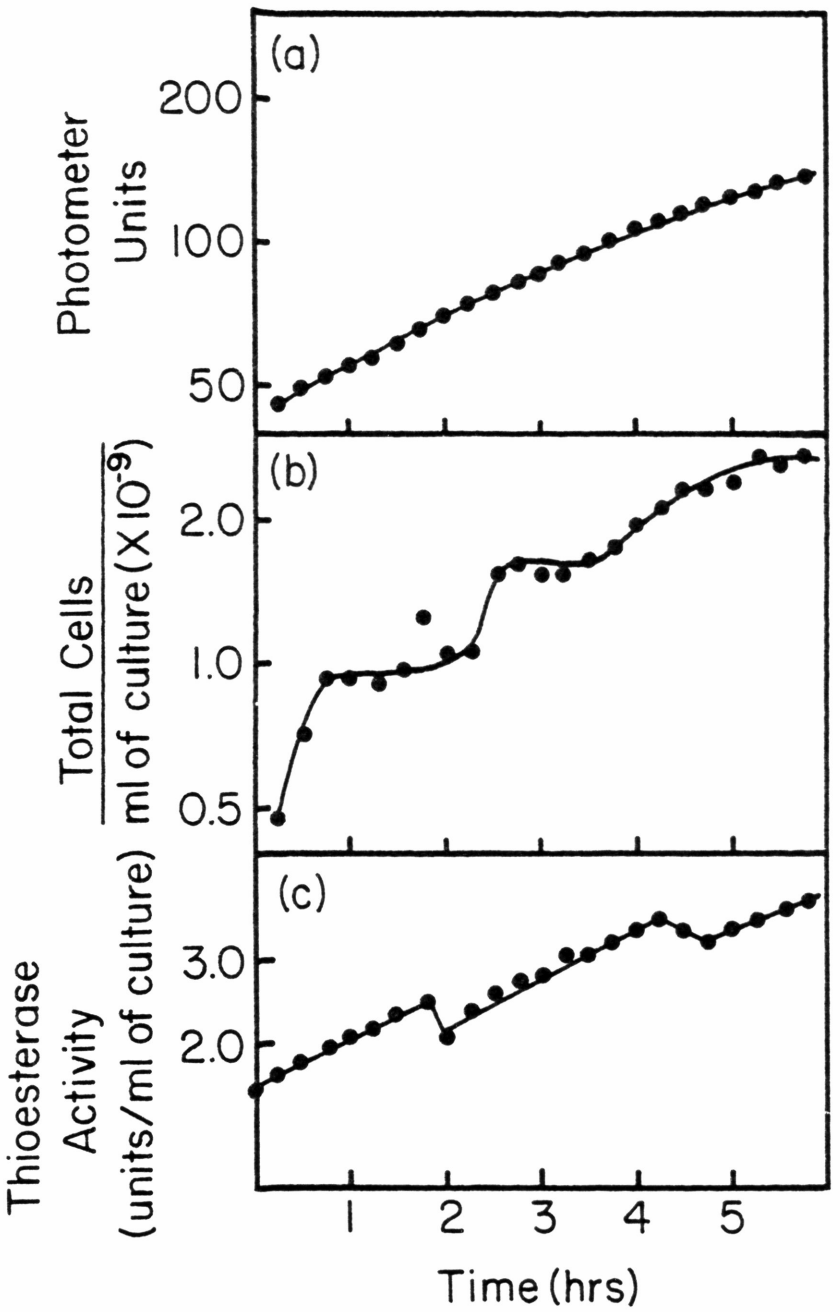

500 ft-c
50 ft-c
Photometer Units
100
50
30
Total Cells / ml of culture (X10^9)
2.0
1.0
Units of Activity / ml of culture
2.5
2.0
1.5
1
2
3
4
5
Time (hrs)

Figure 9 -- Thioesterase activity in synchronously dividing cultures. The synchronization technique is described under "Materials and Methods." a, photometer units; b, total cells per ml of culture; c, thioesterase activity units per ml of culture.


(a)
(b)
(c)
Photometer Units
200
100
50
Total Cells / ml of culture (X 10⁻⁹)
2.0
1.0
0.5
Thioesterase Activity (units/ml of culture)
3.0
2.0
1
2
3
4
5
Time (hrs)

Discussion

Fatty acid synthesis in those systems examined occurs by the successive condensation of two-carbon units ultimately resulting in the production of a sixteen to eighteen carbon fatty acid. In both eukaryotic and prokaryotic organisms, the fatty acids produced are covalently linked by a thioester linkage to a component of the organism's fatty acid synthetase complex.

In mammalian systems, following the completion of fatty acid synthesis, the fatty acid presumably is released from the fatty acid synthetase complex by an enzyme termed thioesterase, which hydrolyzes the thioester bond joining the fatty acid to the synthetase complex. The result of this hydrolysis is the formation of free fatty acids which are then reactivated to their CoA thioester form before subsequently being utilized for complex lipid synthesis. Thus, the mode of fatty acid and phospholipid synthesis in mammalian systems suggests a rationale for the occurrence of thioesterase activity in these systems.

In bacterial systems, the intermediates and products of fatty acid synthesis also occur covalently attached to a component of the fatty acid synthetase complex. In this instance, however, the fatty acids are covalently attached to a low molecular weight protein termed acyl carrier protein (ACP) which serves to stabilize the fatty acids as they are being synthesized.

In bacterial systems, the fatty acid-ACP portion of the complex is known to be completely dissociable from the rest of the complex and is capable of directly serving as the acyl donor for subsequent lipid synthesis, prior hydrolysis and reactivation of the fatty acid to its coenzyme A thioester form not being required (1). Thus, a physiological rationale for thioesterase activity in bacterial systems is not immediately apparent even though these enzymes have been shown to be present and highly active in prokaryotic organisms (2).

In bacterial systems, newly synthesized fatty acids are utilized solely for the purpose of membrane phospholipid synthesis. Thus, by monitoring thioesterase activity under conditions of varying demands for cellular phospholipids, it should be possible to tentatively ascribe an *in vivo* function to the bacterial thioesterases.

The organism utilized for the present study is the nonsulfur, purple, photosynthetic bacterium, *Rhodopseudomonas sphaeroides*. This organism possesses, in addition to a cytoplasmic and outer membrane common to all gram-negative bacteria, an extensive intracytoplasmic membrane system which houses the light harvesting pigments and electron transport components necessary for photosynthetic growth. The presence of this intracytoplasmic membrane is advantageous for the present study because the quantity of internal membrane, as well as the point at which membrane constituents such as phospholipid are produced, can be easily manipulated experimentally using synchronous cell populations and various growth regimens. Therefore, by carefully selecting various growth conditions (and thus demand for internal membrane) one can

monitor the level of thioesterase activity and determine if this activity varies coordinately with the cells demand for internal membrane and its constituent phospholipids. The utility of employing Rhodopseudomonas sphaeroides for studies dealing with the mode and regulation of membrane biogenesis has been thoroughly described earlier by Kosakowski and Kaplan (10).

Thioesterase activity monitored in asynchronously dividing cultures showed that the activity increased proportional to cell growth in cells growing anaerobically at a light intensity of 500 ft-c. Extrapolations of this type, although useful, are not necessarily definitive since culture growth must be viewed cautiously due to the wide distribution of cell ages in asynchronously dividing bacterial populations. The average age of a cell in an asynchronously growing culture is represented by a cell 47% of the way through its division cycle (5).

It is known that membrane content (and thus cellular phospholipid content) is inversely proportional to the light intensity employed for photosynthetic growth. Previous studies have indicated that a change in light intensity from 500 to 50 ft-c. results in many changes in cellular physiology, in particular a decrease in phospholipid production. Thus, one would expect that if the thioesterase was involved in phospholipid synthesis a similar decrease in thioesterase activity would be observed. As predicted, there was a notable decrease in thioesterase activity at approximately the same time there was a marked increase in cell number, suggesting that thioesterase is

probably regulated by cellular processes occurring at cell division.

In order to determine if these changes in thioesterase activity corresponded to periods of cell division, thioesterase activity was monitored in synchronously dividing cultures. The cells were synchronized according to the light shift procedure of Lueking and Campbell (4). In support of the observations made during the light shift, thioesterase activity showed a marked decrease in activity immediately prior to cell division which has also previously been shown to be the point at which maximum phospholipid synthesis occurs (9). These observations suggest that the modulation of thioesterase activity described is in some way related to the cellular division process and possibly to phospholipid (and thus membrane) synthesis. However, the elucidation of the precise in vivo function of this enzyme must await further investigation.

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