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Long-Chain Fatty Acid Assimilation by *Rhodopseudomonas sphaeroides*

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Exogenously supplied long-chain fatty acids have been shown to markedly alleviate the inhibition of phototrophic growth of cultures of *Rhodopseudomonas sphaeroides* caused by the antibiotic cerulenin. Monounsaturated and polyunsaturated C_{18} fatty acids were most effective in relieving growth inhibition mediated by cerulenin. Medium supplementation with saturated fatty acids (C_{14} to C_{18}) failed to influence the inhibitory effect of cerulenin. The addition of mixtures of unsaturated and saturated fatty acids to the growth medium did not enhance the growth of cerulenin-inhibited cultures above that obtained with individual unsaturated fatty acids as supplements. Resolution and fatty acid analysis of the extractable lipids of *R. sphaeroides* revealed that exogenously supplied fatty acids were directly incorporated into cellular phospholipids. Cells treated with cerulenin displayed an enrichment in their percentage of total saturated fatty acids irrespective of the presence of exogenous fatty acids. Cerulenin produced comparable inhibitions of the rates of both fatty acid and phospholipid synthesis and was further found to preferentially inhibit unsaturated fatty acid synthesis.

The elaboration of the intracytoplasmic membrane system of the facultatively phototrophic bacterium *Rhodopseudomonas sphaeroides* has been the subject of numerous investigations concerning the mechanism and regulation of membrane assembly (13, 14, 19, 20). In particular, the recent observation that the insertion of intracytoplasmic membrane protein and phospholipid constituents occurs noncoordinately during intracytoplasmic membrane assembly in *R. sphaeroides* has provided considerable impetus for additional studies on the mechanism(s) employed by this organism to regulate the production and assembly of its membrane protein and lipid components (6, 15, 25).

In general, the ability to experimentally manipulate, via the use of mutants or otherwise, an organism's membrane fatty acid composition has been critical to the development of our knowledge of membrane structure, function, and biogenesis (9, 28). This ability, however, has not been available for studies on membrane biogenesis in cells of *R. sphaeroides* or, for that matter, other representatives of the nonsulfur purple group of photosynthetic procaryotes. Thus, the present report constitutes the first demonstration of the experimental modification of the fatty acid composition of cells of *R. sphaeroides*.

Previous investigators have utilized the antibiotic cerulenin, an inhibitor of bacterial fatty acid synthesis (11), to limit or completely inhibit de novo fatty acid synthesis (2, 4, 11, 16, 31). Furthermore, growth inhibition mediated by cerulenin has been shown to be significantly reversed by the addition of appropriate long-chain fatty acids to the culture medium (2, 16, 31). The cell's direct assimilation of exogenous fatty acids circumvents the inhibitory effect of cerulenin and, in addition, offers investigators an experimentally attractive method for achieving the cellular incorporation of selected fatty acids.

Treatment of *R. sphaeroides* with cerulenin has previously been shown to inhibit (>95%) cellular phospholipid synthesis (4); in the present study, it is shown that the inhibitory effect of cerulenin on phototrophically growing *R. sphaeroides* is markedly alleviated by the addition of long-chain fatty acids to the growth medium. It is also shown that the exogenous fatty acids which restore the growth of cerulenin-inhibited cultures are directly assimilated into the cell's membrane phospholipid fraction.

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MATERIALS AND METHODS

Organism and conditions of growth. *R. sphaeroides* strain M29-5 (Leu^- Met^-), derived from strain 2.4.7, was obtained from Samuel Kaplan, University of Illinois. Unless otherwise stated, the succinic acid minimal medium described by Lueking et al. (24)

containing 0.1% (wt/vol) Brij 58 (polyoxyethylene 20 cetyl ether) detergent was employed for growth. The presence of 0.1% (wt/vol) Brij 58 in the growth medium had no effect upon the phototrophic growth of *R. sphaeroides*. Fatty acid supplements were added in the form of potassium salts solubilized in 0.1% (wt/vol) Brij 58. Cerulenin was stored at −20°C as an ethanolic solution (1 mg/ml) and was added directly to sterile medium. Stock cultures were maintained at −20°C in minimal medium adjusted to 10% (wt/vol) glycerol. Incubations were conducted anaerobically in the light at 32°C with saturating illumination (5,380 lx) provided by a bank of Lumiline lamps (Sylvania). Culture growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter. A value of 100 photometer units corresponds to a culture dry weight of 279 μg/ml and a cell density of approximately 10^9 cells per ml (24).

Lipid extraction and fatty acid analysis. Cells utilized for fatty acid analyses were harvested by centrifugation and freed of residual, unincorporated fatty acids by washing three times in 0.01 M KH_2PO_4 buffer (pH 7.0) containing 0.1% (wt/vol) Brij 58. Cellular lipids were extracted by the method of Bligh and Dyer (3) as described by Ames (1). Phase partitioning and washing of the chloroform extracts was conducted as described by Lueking et al. (24). The washed chloroform extracts were applied to columns of silicic acid equilibrated in chloroform-methanol (49:1, vol/vol), and neutral lipids and photopigments were removed by elution of the column with 10 column volumes of this same chloroform-methanol mixture (18). A polar lipid fraction, consisting of cellular phospholipids (8), was then obtained by elution of the column with 10 column volumes of methanol.

Fatty acid methyl esters were prepared by direct transesterification of phospholipid acyl moieties. Transesterifications were conducted for 4 h at 79°C in methanolic HCl (2 ml) prepared by the addition of 2.5 ml of acetyl chloride to 25 ml of cold, anhydrous methanol. After transesterification, 5 ml of a 5% (wt/vol) solution of NaCl was added to the transesterification mixture, and the fatty acid methyl esters were extracted three times with 3 ml of hexane. The hexane fractions were pooled, washed with 4 ml of 2% (wt/vol) potassium bicarbonate, and concentrated under nitrogen, and the fatty acid methyl esters were purified by thin-layer chromatography on plates of silica gel G developed in hexane-diethyl ether (9:1, vol/vol) (7). The plates were sprayed with 2,7-dichlorofluorescein, and the methyl esters were visualized under UV light and eluted with chloroform. The purified fatty acid methyl esters were stored at −20°C under an atmosphere of nitrogen.

Compositional analysis of the purified fatty acid methyl esters was conducted with a Perkin-Elmer 900 gas-chromatograph equipped with a hydrogen flame ionization detector. Separations were obtained on a column (1.83 m by 3.2 mm) of 10% diethylene glycol succinate on 80/100 Supelcoport (Supelco, Bellefonte, Pa.) operated at 170°C. Individual fatty acids were identified by comparison of retention times with authentic standards, and the proportions of individual components were calculated by multiplying peak height by retention time (17, 21).

In vivo rates of fatty acid synthesis. Cellular rates of fatty acid synthesis were determined by pulse-labeling with [^{14}C]acetate. Cells (4.5 ml) adapted to growth (four to six mass doublings) in the designated media were transferred to 4.7-ml screw-capped vials containing 1 μCi of [^{14}C]acetate (55 μCi/μmol) and were incubated for 8 min under saturating illumination (5,380 lx). Under these conditions, the incorporation of [^{14}C]acetate into the cells' extractable lipid fraction was linear with time (8 min), and >90% of the incorporated radioactivity was localized in the polar lipid fraction. Upon completion of the pulsing period, culture samples (0.60 ml) were combined with 150 μg of carrier phospholipid, and the cellular lipids were extracted and fatty acid methyl esters were prepared and purified as described above. Unsaturated and saturated fatty acid methyl esters were resolved by argentation chromatography on plates of silica gel G impregnated with 10% silver nitrate as described by Cubero and Mangold (10). Methyl esters were localized by spraying the plates with 2,7-dichlorofluorescein and were eluted directly into scintillation vials with chloroform. After evaporation of the chloroform, the radioactive methyl esters were quantitated by scintillation counting in a toluene-based scintillant. Control studies showed that, after transesterification of the radioactive polar lipid fraction, 88% of the recoverable radioactivity was localized in the combined hexane extracts and 96% of the hexane extractable material comigrated with authentic fatty acid methyl esters during chromatography on plates of silica gel G as described above.

Rates of cellular phospholipid synthesis. Rates of cellular phospholipid synthesis were determined by monitoring the incorporation of [^{32}P]orthophosphoric acid into the cellular lipid fraction. Cells utilized for ^{32}P labeling were extensively adapted to growth in a modified, low-phosphate (2 mM phosphate 20 mM morpholinopropanesulfonic acid–succinic acid–Brij 58 minimal medium. Pulse-labeling with [^{32}P]orthophosphoric acid was conducted exactly as described by Fraley et al. (15), with the exception that morpholinopropanesulfonic acid buffer was substituted for the Tris buffer in the low-phosphate medium (12).

Phospholipid compositional analysis. Cells employed for the determination of phospholipid compositions were grown for six mass doublings in the low-phosphate, succinic acid–Brij 58 medium described above that contained 10 μCi of carrier-free [^{32}P]orthophosphoric acid per ml. Samples (1.4 ml) of these cultures were then transferred to tubes containing carrier cells (150 μg of phospholipid), and the cellular lipids were extracted as described above. All extractions and analyses were performed in duplicate. Individual phospholipid species were separated by two-dimensional, thin-layer chromatography on plates of boric acid-impregnated silica gel G as described by Cain et al. (6). Phospholipids were localized by autoradiography and iodine staining, and the areas of the plates containing labeled phospholipids were then scraped into scintillation vials and quantitated by scintillation counting in a toluene-based scintillation cocktail.

Chemicals. Fatty acids (99% pure) and cerulenin (purified 95% from *Cephalosporium caerulens*) were obtained from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]acetate (55 mCi/mmol), uniformly labeled [^{3}H]acetate (80 mCi/mmol), and carrier-free [^{32}P]orthophosphoric acid were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals were of reagent grade or better.

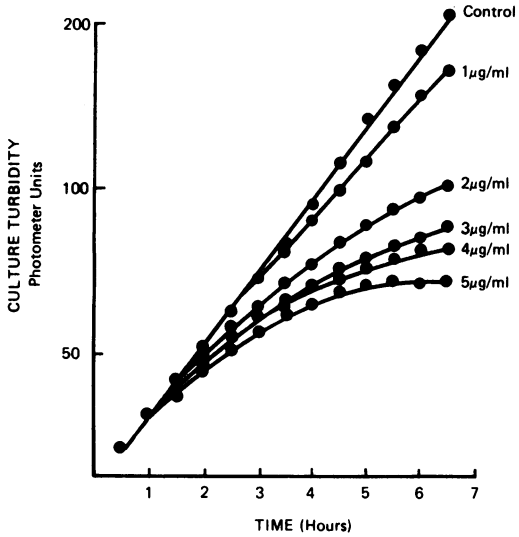

FIG. 1. Influence of cerulenin on the phototrophic growth of *R. sphaeroides*. Cells adapted to logarithmic-phase growth (anaerobic, light) in succinic acid minimal medium were subcultured into this same medium containing the designated levels of cerulenin. Culture growth was monitored as described in the text.

RESULTS

Influence of exogenously supplied fatty acids upon cerulenin-mediated growth inhibition. In agreement with Broglie and Niederman (4), cerulenin was found to effectively inhibit the phototrophic growth of *R. sphaeroides*. Significant inhibition (70 to 75%) of culture growth was observed at a cerulenin concentration of 2 μg/ml, and culture growth was completely inhibited at a cerulenin concentration of 5 μg/ml (Fig. 1). As is characteristic of cerulenin action (5), 1 to 1.5 culture mass doublings were required before the inhibitory effect of this antibitoic was fully expressed.

Studies with *Escherichia coli* have shown that growth inhibition caused by high levels of cerulenin can be overcome by the addition of both saturated and unsaturated fatty acids to the growth medium (16). In contrast, no individual fatty acids or combination of fatty acids examined were found to be effective in even partially alleviating a complete inhibition of cell growth of *R. sphaeroides* caused by cerulenin. However, by titrating the concentration of cerulenin in the presence of a constant amount (20 μg/ml) of individual fatty acids it was observed that some fatty acids significantly stimulated (2.6-fold) cell growth in the presence of growth-limiting cerulenin levels. The stimulatory effect of linoleic acid (18:2) upon cerulenin-treated cultures of *R. sphaeroides* is shown in Fig. 2. Also, results virtually identical to those presented in Fig. 2 were obtained when *cis*-vaccenic (18:1), oleic (18:1), or linolenic acid (18:3) was substituted for linoleic acid (data not shown). In all instances the stimulatory effect of the fatty acids was independent of the fatty acid concentration over a range of 20 to 100 μg of fatty acid per ml.

Comparison of the data presented in Fig. 1 and 2 shows that linoleic acid is able to completely reverse the growth inhibition caused by 1 μg of cerulenin per ml. However, the greatest stimulation of growth was observed in fatty acid-supplemented cultures that contained 2 μg of cerulenin per ml. (Fig. 2). Importantly, at cerulenin levels greater than 2 μg/ml the stimulatory effect of exogenous fatty acids was either negligible or absent. For these reasons, a cerulenin concentration of 2 μg/ml was selected for use in further studies.

Medium supplementation with palmitic (16:0), stearic (18:0), myristic (14:0), or myristoleic (14:1) acid failed to stimulate cell growth above that observed in cerulenin-treated (2 μg/ml) cultures that did not contain fatty acids (Fig. 3). The growth stimulation produced by palmitoleic acid (16:1) (Fig. 3) was small and not consistently observed.

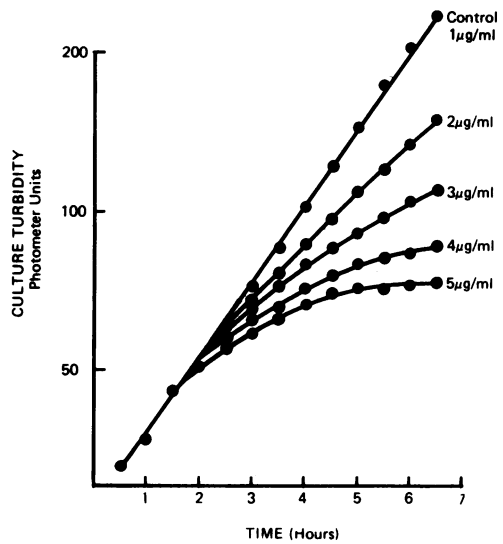

FIG. 2. Effect of exogenous linoleic acid on the pattern of cerulenin inhibition of phototrophically growing *R. sphaeroides*. Cells adapted to logarithmic-phase growth in minimal medium were used to inoculate medium supplemented with 20 μg of linoleic acid per ml and the designated concentration of cerulenin. Cerulenin was omitted in the control culture. Cultures containing 1 μg of cerulenin per ml gave results identical to those of the control cultures. Cell growth was monitored as described in the text. Identical results were obtained when *cis*-vaccenic, oleic, or linolenic acid was substituted for linoleic acid. All fatty acids were supplemented at 20 μg/ml.

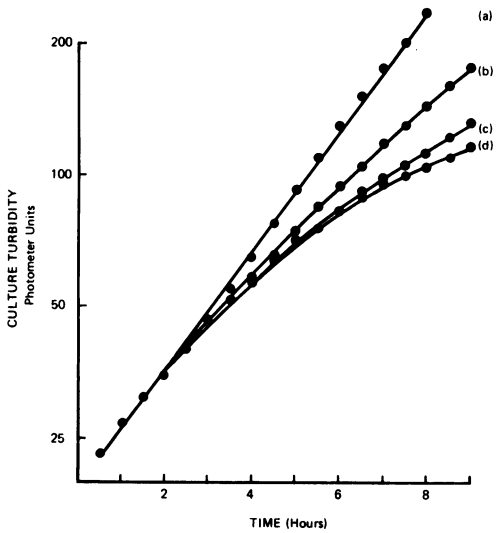

FIG. 3. Effect of various fatty acids upon the growth of *R. sphaeroides* in the presence of cerulenin. Cells adapted to logarithmic-phase growth were used to inoculate fatty acid-supplemented media containing 2 μg of cerulenin per ml. Growth was monitored turbidimetrically as described in the text. All fatty acids were present at 20 μg/ml. (a) control culture lacking fatty acids and cerulenin; (b) response observed upon supplementation with linoleic, *cis*-vaccenic, oleic, or linolenic acid in the presence of cerulenin; (c) palmitoleic acid plus cerulenin; (d) cerulenin control minus fatty acid, and cerulenin plus myristic, myristoleic, palmitic, or stearic acid.

The stimulation of cell growth observed with C_{18} unsaturated fatty acids was not a transient phenomenon. Those fatty acids which produced a marked stimulation of growth of cerulenin-inhibited cells continued to provide their stimulatory effect for at least six to eight mass doublings. Thus, cells adapted (three mass doublings) to growth in cerulenin- and fatty acid-containing media continued to grow for three generations when subcultured into the same medium (Fig. 4). Interestingly, supplementation of cerulenin-inhibited cultures with mixtures of unsaturated and saturated fatty acids did not enhance growth above that obtained when individual unsaturated fatty acids were employed as supplements (Fig. 4). Instead, the saturated fatty acids investigated were, by themselves, growth inhibitory (data not shown) and also diminished the growth stimulation generally observed with the individual unsaturated fatty acids as the sole supplements (Fig. 4).

Assimilation of exogenous long-chain fatty acids. The finding that the fatty acid pseudoauxotrophy resulting from the addition of growth-limiting quantities of cerulenin to cultures of *R. sphaeroides* could only be satisfied by medium supplementation with C_{18} monounsaturated and polyunsaturated fatty acids is remarkably analogous to the results reported by Buttke and Ingram (5) concerning the effects of low concentrations of cerulenin on the growth of *E. coli*. These investigators clearly showed that, in *E. coli*, low concentrations of cerulenin selectively inhibit unsaturated fatty acid synthesis and that this inhibition can only be relieved by exogenously supplied unsaturated fatty acids.

To confirm that *R. sphaeroides* directly utilized exogenously supplied unsaturated fatty acids to negate the inhibitory effect of cerulenin, fatty acid compositional analyses were conducted upon cellular phospholipid fractions prepared from cells that had been extensively adapted (six mass doublings) to growth in media containing cerulenin and various exogenous fatty acids. Lipid extractions and fatty acid analyses were conducted as described above. The results of these studies are summarized in Table 1.

Control cultures grown in minimal medium containing 0.1% (wt/vol) Brij 58, but lacking cerulenin and exogenous fatty acids, possessed the distinctive fatty acid profile previously reported for phototrophically grown cells of *R. sphaeroides* (Table 1) (22). Thus, *cis*-vaccenic acid constituted approximately 90% of the total fatty acids, with the remaining 10% being com-

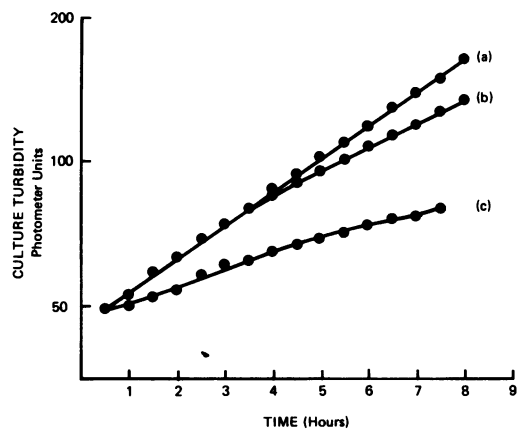

FIG. 4. Comparison of the efficacy of individual fatty acids in the maintenance of cell growth in the presence of cerulenin. Cells were adapted for three mass doublings in medium supplemented with individual fatty acids and 2 μg of cerulenin per ml. Individual cultures were then subcultured into media of identical compositions, and cell growth was monitored as described in the text. Palmitic acid was present at 10 μg/ml. All other fatty acids were added at a concentration of 20 μg/ml. (a) Cerulenin plus linoleic, linolenic, *cis*-vaccenic, or oleic acid; (b) cerulenin plus linoleic (20 μg/ml) and palmitic (10 μg/ml) acids; (c) cerulenin only.

TABLE 1. Effect of cerulenin and exogenous fatty acids on the acyl group composition of the total cellular phospholipids of *R. sphaeroides*

Fatty acid[a]	Fatty acid analysis[b] with the following additions to medium:							
	None	Cerulenin[c] plus:						
		No fatty acid	$C^{\Delta 9,12}_{18:2}$	$C^{\Delta 9,12,15}_{18:3}$	$C^{\Delta 9}_{18:1}$	$C^{\Delta 11}_{18:1}$	$C^{\Delta 9,12}_{18:2}/C_{16:0}$	$C^{\Delta 9,12}_{18:2}$
Saturated								
14:0	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
16:0	3.4	24.4	12.6	17.6	12.9	11.4	19.8	2.6
18:0	6.9	14.9	12.9	14.5	16.3	12.4	12.5	5.9
Total	10.3	39.3	25.5	32.1	29.2	23.8	32.3	8.5
Unsaturated								
14:1	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
16:1	0.7	2.2	0.9	1.6	1.0	1.4	1.3	Trace
18:1	88.5	58.0	49.1	44.8	69.1	74.4	54.9	69.2
18:2			29.0				12.8	21.7
18:3				18.5				
Total	89.2	60.2	79.0	64.9	70.1	75.8	69.0	90.9

[a] Fatty acids were added at a concentration of 20 μg/ml, except for 16:0 which was added at a concentration of 10 μg/ml.

[b] All fatty acid analyses were performed in duplicate upon samples obtained from at least two separate experiments. Values are presented as weight percents.

[c] Cerulenin was added at a concentration of 2 μg/ml.

prised of small quantities of stearic, palmitic, myristic, and palmitoleic acids.

Irrespective of the presence or absence of exogenous fatty acids, cell cultures treated with cerulenin displayed an elevation in their total percentage of saturated fatty acids. In the absence of exogenous fatty acid, cerulenin treatment resulted in an almost fourfold increase in the percentage of saturated fatty acids and a 35% reduction in the cellular level of *cis*-vaccenic acid. In the combined presence of cerulenin and exogenous fatty acids the percentage of saturated fatty acids increased 2.8-fold (average), with palmitic acid typically displaying the greatest individual increase among the saturated fatty acids (Table 1).

Importantly, linoleic and linolenic acids, which are not produced by *R. sphaeroides* but which were shown to stimulate the growth of cerulenin-inhibited cultures, were found to be directly esterified into cellular phospholipids. Incorporations observed with linoleic and linolenic acids were 29 and 18.5% of the total phospholipid acyl groups, respectively.

The occurrence and extent of exogenous fatty acid assimilation was also monitored in the absence of cerulenin (Table 1). Although linoleic acid assimilation occurred under these conditions, the extent of its incorporation and the reduction in cellular 18:1 fatty acid content were less than those observed in the presence of cerulenin. Also, fatty acid supplementation in the absence of cerulenin had essentially no effect, qualitatively or quantitatively, upon the cells saturated fatty acid profile.

Influence of cerulenin on the in vivo rates of phospholipid and fatty acid synthesis. Since the cerulenin concentration (5 μg/ml) required to completely inhibit the growth of *R. sphaeroides* in the present study was much less than that (20 μg/ml) reported by Broglie and Niederman (4), studies were conducted to determine whether the extent of growth inhibition caused by cerulenin accurately reflected the degree of inhibition of cellular lipid synthesis. The data presented in Table 2 show the effect of cerulenin upon the rate of cellular phospholipid synthesis. Rates of phospholipid synthesis were measured by pulse-labeling with $^{32}P_i$ as described above.

The rate of phospholipid synthesis was inhibited 75% in cultures containing 2 μg of cerulenin per ml and was almost completely inhibited (95%) by a cerulenin concentration of 5 μg/ml (Table 2). Thus, these data (Table 2) clearly show that the observed inhibitory effect of cerulenin on culture growth rate (Fig. 1, 2, and 3) directly reflect its inhibitory effect on the rate of cellular phospholipid synthesis. Furthermore, the rates of both culture growth (Fig. 3) and phospholipid synthesis (Table 2) were stimulated 2.6-fold by the addition of *cis*-vaccenic acid to cerulenin (2 μg/ml)-inhibited cultures. The rate of phospholipid synthesis was not affected in cultures which contained only exogenous *cis*-vaccenic acid (Table 2).

As mentioned above, *R. sphaeroides* and *E.*

TABLE 2. Effect of cerulenin and exogenous fatty acids upon the rate of cellular phospholipid synthesis

Additions to medium	Rate of phospholipid synthesis[a] (cpm/100 μg dry wt/17 min)	% Inhibition
None[b]	26,263	0
Cerulenin (2 μg/ml)[c]	6,497	75
Cerulenin (2 μg/ml) and *cis*-vaccenic acid (2 μg/ml)	16,930	35
cis-Vaccenic acid (20) μg/ml)	27,687	0
Cerulenin (5 μg/ml)	1,425	95

[a] Samples (4.5 ml) of cultures in the midlogarithmic phase of growth (80 to 100 photometer units) were transferred to 4.7-ml screw-capped vials containing 250 μCi of carrier-free [^{32}P]orthophosphoric acid and were incubated under saturating illumination for 17 min. Samples (0.6 ml) were monitored for ^{32}P incorporation into cellular lipids. Lipid extraction and analysis were as described in the text.

[b] Control cells were adapted to growth in the low-phosphate, succinic acid–Brij 58 minimal medium for six mass doublings.

[c] Cells were grown for 6 h after the addition of cerulenin before pulse-labeling with $^{32}P_i$ (Fig. 1).

coli display similar responses to the presence of low concentrations of cerulenin in the culture medium. The growth of both organisms is only stimulated by unsaturated fatty acids, and both display characteristic alterations in the relative percentages of their saturated fatty acids. In addition, Buttke and Ingram (5) convincingly demonstrated that the unsaturated fatty acid auxotrophy conferred upon *E. coli* by low levels of cerulenin resulted from a preferential inhibition of the fatty acid biosynthetic enzyme, β-ketoacyl-acyl carrier protein synthetase I and, interestingly, was typically accompanied by a stimulation of saturated fatty acid synthesis.

To determine whether the similarities in response to cerulenin displayed by *E. coli* and *R. sphaeroides* extended to the differential effect of cerulenin upon the in vivo rates of saturated and unsaturated fatty acid synthesis, the influence of cerulenin upon the rates of synthesis of these two classes of fatty acids in *R. sphaeroides* was examined. In vivo rates of fatty acid synthesis were determined by pulse-labeling with [^{14}C]acetate as described above.

The data presented in Table 3 show that the apparent (uncorrected) inhibition (45%) of the rate of fatty acid synthesis by cerulenin (2 μg/ml) was significantly lower than the inhibition (75%) of phospholipid synthesis observed under identical conditions (Table 2). However, since these data (Tables 2 and 3) were obtained with cells adapted to steady-state growth in cerulenin-containing media, it was proposed that the observed discrepancy was due to a cerulenin-induced constriction of the endogenous acetate pool. Such a constriction would result in an increased specific activity of the [^{14}C]acetate precursor pool, which would erroneously increase the value of [^{14}C]acetate incorporation.

To evaluate this proposal, control cultures and cultures containing 2 μg of cerulenin per ml were continuously labeled by growth (six mass doublings) in low-phosphate medium containing both [^{3}H]acetate (5 μCi/ml) and $^{32}P_i$ (4 μCi/ml). After extraction of the cellular lipids, polar lipid fractions were prepared (see above), and the radioactivity contained in these fractions was quantitated. As predicted, the polar lipid fraction obtained from the control culture displayed

TABLE 3. Effect of cerulenin and exogenous fatty acids on the in vivo rate of fatty acid synthesis

Additions to medium[a]	Rate of total fatty acid synthesis (cpm/100 μg dry wt/8 min)[b]		% Inhibition		Rate of fatty acid synthesis (cpm/100 μg dry wt/8 min)[c]	
	Uncorrected	Corrected	Uncorrected	Corrected	Unsaturated	Saturated
None	28,317	28,317	0	0	27,184 (96)	1,133 (4)
Cerulenin (2 μg/ml)	15,657	4,691	44.7	83.4	3,378 (72)	1,313 (28)
Cerulenin (2 μg/ml) and *cis*-vaccenic acid (20 μg/ml)	13,479	4,039	52.4	85.7	2,948 (73)	1,091 (27)
Cerulenin (5 μg/ml)	3,992	1,193	86.0	95.7	549 (46)	644 (54)

[a] The basal medium was as described in footnote *b* of Table 2.

[b] The rates of fatty acid synthesis were determined as described in the text. The uncorrected values represent the total quantities of [^{14}C]acetate incorporated into the cellular fatty acid fraction under the designated conditions. Corrected values, normalized to the control value, have been adjusted by considering the apparent 3.3-fold increase in [^{14}C]acetate specific activity which occurs in the presence of cerulenin (see the text).

[c] The rates of synthesis of unsaturated and saturated fatty acids, corrected for recoveries, were determined as described in the text. The values within parentheses are the percent distributions of ^{14}C counts per minute recovered in unsaturated and saturated fatty acids.

a $^3H/^{32}P$ (counts per minute) ratio of 0.95, whereas the equivalent fraction obtained from cells treated with cerulenin displayed a $^3H/^{32}P$ ratio of 3.17. Thus, an apparent 3.3-fold increase in the specific activity of the [^{3}H]acetate precursor pool occurred in those cells grown in the presence of 2 μg of cerulenin per ml. Moreover, when this change in acetate specific activity was considered during calculations of the rates of fatty acid synthesis, the corrected values (Table 3) for the percent inhibition of fatty acid synthesis were in good agreement (8 to 10%) with those values previously obtained for the inhibition of phospholipid synthesis (Table 2) and cell growth (Fig. 1 and 3). The $^3H/^{32}P$ ratio of the polar lipid fraction obtained from cells labeled (as above) in media containing 2 μg of cerulenin per ml and 20 μg of *cis*-vaccenic acid per ml was 3.4-fold lower (0.93) than the $^3H/^{32}P$ ratio of 3.17 observed for cells treated with cerulenin alone. This reduction was primarily due to the 2.6-fold stimulation of phospholipid synthesis produced by the exogenous, unlabeled fatty acid supplement.

To determine the effect of cerulenin on the relative rates of unsaturated and saturated fatty acid synthesis, the radioactive fatty acids present in the polar lipid fractions of cells pulse-labeled with [^{14}C]acetate were converted to their fatty acid methyl esters, purified, and resolved by argentation chromatography as described above. Cerulenin was found to preferentially inhibit the synthesis of unsaturated fatty acids (Table 3). A significant influence of cerulenin on the rate of saturated fatty acid synthesis was only observed at a cerulenin concentration (5 μg/ml) which caused a 95% inhibition of both fatty acid and phospholipid synthesis. This result is in marked contrast of that reported by Buttke and Ingram (5) for *E. coli*, where a large stimulation of saturated fatty acid synthesis was observed at low concentrations of cerulenin. The present results are further supported by the close agreement (±10%) between the values for the percentage (of total) rates of saturated and unsaturated fatty acid synthesis (Table 3) and the fatty acid composition data presented in Table 1. The presence of cerulenin or fatty acids (or both) in the growth medium had little influence upon the polar head group distributions of the cellular phospholipids (Table 4). However, a slight increase (~6%) in phosphatidylglycerol and a slight decrease (~4%) in phosphatidylcholine were routinely observed in cells treated with 2 μg of cerulenin per ml. *N*-Acylphosphatidylserine, a novel phospholipid recently identified in cells of *R. sphaeroides* by Donohue et al. (12), comprised <1% of the total cellular phospholipids under all growth conditions examined. Thus, morpholinopropanesulfonic acid buffer appears to be an acceptable substitute for Tris buffer (which promotes *N*-acylphosphatidylserine accumulation) in the low-phosphate medium employed for radioactive labeling of the phospholipids of *R. sphaeroides*.

TABLE 4. Effect of cerulenin and exogenous fatty acids on the cellular phospholipid composition of *R. sphaeroides*

Additions to medium[a]	Phospholipid composition (%)[b]		
	PE	PG	PC
None	43.4	30.8	13.6
Cerulenin (2 μg/ml)	42.8	34.5	11.3
Cerulenin (2 μg/ml) and *cis*-vaccenic acid (20 μg/ml)	42.1	37.0	9.1
cis-Vaccenic acid (20 μg/ml)	42.2	30.0	14.4

[a] Cells were grown for six mass doublings in the designated medium containing 10 μCi of [^{32}P]orthophosphoric acid per ml. Cellular phospholipids were extracted and quantitated as described in the text.

[b] The values are presented as the percentage of total lipid-extractable radioactivity contained in the individual phospholipid species. All determinations were performed in duplicate.

DISCUSSION

Previous studies (5, 23) employing the nonsulfur purple photosynthetic bacteria have included the use of cerulenin to inhibit phospholipid synthesis; however, these studies provided no evidence concerning the specificity of the observed inhibition. In this regard, the present study shows that exogenously supplied fatty acids are able to relieve a growth inhibition of *R. sphaeroides* caused by cerulenin, but the ameliorative effect of the fatty acids is conditional. The growth-stimulatory effect of added fatty acids is only realized at growth-limiting concentrations of cerulenin and only when C_{18} unsaturated fatty acids are employed as supplements. When cell growth in liquid culture is completely inhibited by cerulenin, then medium supplementation with fatty acids is ineffectual in even partially reversing the inhibition. Accordingly, this observation suggests that the influence of cerulenin upon the growth of *R. sphaeroides* may extend, either directly or indirectly, beyond its known function as an inhibitor of fatty acid synthesis.

The possibility of a direct involvement of cerulenin in the limitation of photopigment production by *R. sphaeroides* was considered by Broglie and Niederman (5), but not thoroughly pursued. These investigators concluded that the curtailment of photopigment production they observed in the presence of cerulenin resulted secondarily upon a primary limitation of phos-

pholipid biosynthesis. As is suggested by the present study, however, this conclusion may be compromised since it was not shown that the cerulenin-mediated inhibition of phospholipid synthesis was recoverable by exogenous fatty acids. For example, it would be informative to determine whether the inhibitions of cell growth, phospholipid synthesis, and photopigment production observed in the presence of cerulenin by Broglie and Niederman (5) could be coordinately alleviated by fatty acid supplementation of the growth medium.

The basis for the inability of exogenous fatty acids to reverse a complete inhibition of growth of *R. sphaeroides* is unknown. However, a similar observation was made by Rottem et al. (29) during studies on the effect of cerulenin upon the growth of *Proteus mirabilis*. These investigators found that the ability of exogenous fatty acids to overcome a cerulenin-mediated inhibition of growth decreased markedly at elevated cerulenin concentrations, and they attributed this finding to the organism's inability to produce sufficient quantities of hydroxy fatty acids. Support for this proposal was obtained by demonstrating a 30 to 50% decrease in lipopolysaccharide content of *P. mirabilis* cells that were treated with cerulenin. In this regard, studies (Campbell and Lueking, unpublished observations) involving the phototrophic growth of *R. sphaeroides* on solidified medium indicate that, under these conditions, exogenous fatty acids can reverse a complete inhibition of cell growth caused by cerulenin. The molecular basis for this observation is currently being investigated.

The specific requirement for unsaturated fatty acids in relieving growth inhibition is consistent with the observed preferential inhibition of unsaturated fatty acid synthesis (Table 3) and is not surprising in view of the high content (90%) of unsaturated fatty acids normally possessed by *R. sphaeroides* (12, 22). However, it is not clear whether the ineffectiveness of saturated fatty acids in stimulating growth reflects the organism's physiological requirements or assimilatory capability. In any event, a requirement for both saturated and unsaturated fatty acids would not necessarily be observed in the growth-limited, rather than completely inhibited, cultures utilized for the present study. Under the growth conditions employed, *R. sphaeroides* was able to satisfy its requirement for saturated fatty acids via endogenous synthesis and, in fact, appeared to prefer an endogenous rather than an exogenous origin for these fatty acids. Thus, in addition to the failure of saturated fatty acids to stimulate growth, their presence in the culture medium did not significantly alter the cell's saturated fatty acid profile.

Importantly, the present results, together with other available information (26), cumulatively support the presence of a type II fatty acid synthetase in cells of *R. sphaeroides*. Type II synthetases exhibit an obligate requirement for acyl carrier protein (30) and are inhibited 95% in the presence of 4 to 8 μg of cerulenin per ml (27, 30). Although acyl carrier protein has not been demonstrated in cells of *R. sphaeroides*, its presence is virtually assured by the obligate dependence for acyl-acyl carrier protein substrates displayed by this organism's *sn*-glycerol 3-phosphate acyltransferase (26).

In summary, it has been shown that *R. sphaeroides* is able to directly utilize exogenous fatty acids for cellular lipid synthesis and that this ability can be exploited to obtain cells with modified fatty acid compositions. Further, the use of cerulenin to render the growth of *R. sphaeroides* substantially dependent upon exogenous fatty acids provides an alternative approach to the use of fatty acid auxotrophs (not currently available for *R. sphaeroides*) for studying the mode and regulation of phospholipid synthesis and membrane biogenesis in this organism.

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