Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 126-132

# THE CERULENIN-INDUCED FORMATION OF 1-ACYL-LYSOPHOSPHATIDYL GLYCEROL IN BACILLUS MEGATERIUM

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Received November 23, 1977

SUMMARY: When a culture of Bacillus megaterium ATCC 14581, growing at 20° and treated with the fatty acid synthesis inhibitor, cerulenin, was incubated with [U-$^{14}$C]palmitate, 50% of the incorporated label was found in 1-palmitoyl-lysophosphatidyl glycerol within 5 min. Most of the remaining $^{14}$C appeared in free fatty acid and phosphatidyl glycerol. By 45 min almost all of the lyso compound had disappeared and 80% of the incorporated label was found in phosphatidyl glycerol. At 20°, in the absence of cerulenin or at 35° in either its presence or absence, no labeled lysophosphatidyl glycerol could be found at any time after [U-$^{14}$C]palmitate addition. The major radioactive lipid, in these cases, was always phosphatidyl glycerol. At 20°, the palmitate of phosphatidyl glycerol but not of lysophosphatidyl glycerol was readily desaturated.

Previous publications from this laboratory (1,2) have described the mechanisms by which alterations of environmental temperature mediate changes in the rate of monounsaturated fatty acid synthesis in B. megaterium. Such changes are eventually reflected in adaptive alterations in the fatty acid composition of the membrane lipids (3). We were therefore interested in how fatty acids enter the membrane phospholipids of B. megaterium and whether incorporation is also temperature-mediated. In our initial studies, we sought to determine the effect of temperature on the utilization of added palmitate in the absence of endogenous fatty acid synthesis. To shut down fatty acid synthesis in B. megaterium we selected the antibiotic, cerulenin, which inhibits this process in a large variety of microorganisms, presumably by selectively binding to β-ketoacyl-ACP synthetase (4). In the course of this work, we observed the temperature- and cerulenin-dependent incorporation of [U-$^{14}$C] palmitate into a unique lipid, the appearance and identification of which are the subjects of this article.

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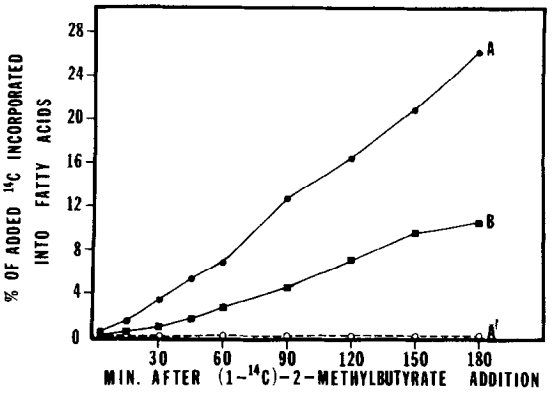



Figure 1. Effect of cerulenin on fatty acid synthesis. Two equal portions from each of 2 cultures growing at 20° were transferred to flasks in a 20°-water-bath shaker when cell densities reached 162 (A) and 305 (B) Klett units. One portion from each culture served as a control while cerulenin (20 μg/ml) was added to each second portion. After 15 min, [1-$^{14}C$]-2-methylbutyrate (30 nmoles/ml) was added to each flask and samples were taken at intervals for the determination of $^{14}C$-incorporation into fatty acids. Label incorporation into fatty acids for culture A in the absence and presence of cerulenin is shown by curves A and A' respectively. Curve B shows $^{14}C$ incorporation, in the absence of cerulenin, for culture B. Incorporation in cerulenin-treated culture B (not shown) was essentially the same as that shown by curve A'.

## MATERIALS AND METHODS

Cerulenin was obtained from Makor Chemicals, Ltd. (Jerusalem). [U-$^{14}C$] palmitic acid (900 μCi/μmole) and the sodium salt of [1-$^{14}C$]DL-2-methylbutyric acid (1.3 μCi/μmole) were purchased from Amersham/Searle Corp. and International Chemical and Nuclear Corp., respectively. Phosphatidyl glycerophosphate was a gift from Dr. William Dowhan. All other lipid standards as well as phospholipase $A_2$ (from bee venom) were purchased from Sigma Chemical Co.

Preparation of 1-acyl-lysophosphatidyl glycerol: L-α-phosphatidyl-DL-glycerol (1 mg) was dissolved in 2 ml of 5% methanol in ethanol, 30 μl of phospholipase $A_2$ solution (5 mg venom per ml of 0.1 M tris-chloride buffer, pH 7.2 and 0.01 M $CaCl_2$) was added and the mixture was incubated with gentle shaking for 10 h at 22 ± 2°. Two ml of ethanol were then added and the resultant clear solution was evaporated to dryness under a stream of $N_2$. The crude product was taken up in $CHCl_3$-$CH_3OH$ (2:1, v/v) and purified by thin-layer silica gel chromatography. Controls, in which phospholipase $A_2$ was absent, showed little evidence of phosphatidyl glycerol hydrolysis.

Extraction of bacteria: After cells were harvested on a glass depth filter (Millipore AP 25), both cells and filter were placed in a vial containing 7.6 ml of $CHCl_3$:$CH_3OH$:$H_2O$ (1:2:0.8, v/v/v) and ruptured by two 30 sec bursts with a Branson Sonifier S-110 equipped with a micro tip. After filtration of the sonicate, the filter disc was washed with 3 ml of the extraction solvent, 3 ml of $H_2O$ and finally 3 ml of $CHCl_3$. The $CHCl_3$ phase was removed and saved and the upper ($H_2O$-$CH_3OH$) phase was extracted 3 times with $CHCl_3$. These combined $CHCl_3$ phases contained all of the bacterial lipids except lipid X (see "Results") which remained in the $H_2O$-$CH_3OH$ phase. When the aqueous methanol

phase was acidified with 2 drops of 6 N HCl, lipid X could then be totally removed by 3 extractions with $CHCl_3$.

All other procedures, including growth and incubation of bacteria, thin-layer chromatography, determination of desaturation of palmitate by the mercuric acetate complex procedure and counting methods have been described previously (1,5).

## RESULTS

Effect of cerulenin on fatty acid synthesis: As Fig. 1 shows, cerulenin at 20 μg/ml completely inhibits fatty acid synthesis from added [1-$^{14}$C]-2-methylbutyrate, a precursor of 12-methyltetradecanoate*, the major fatty acid of B. megaterium. Cerulenin at 10 μg/ml (data not shown) allowed synthesis to proceed at about 4% of the rate obtained in the absence of cerulenin.

Effects of cerulenin on the utilization of [U-$^{14}$C]palmitate: [U-$^{14}$C]palmitate was added to control and cerulenin-treated cultures growing at 20°. The cells were then harvested at intervals and extracted to obtain the lipids. As expected, when the extracts from the control culture were mixed with additional $CHCl_3$ and $H_2O$ to cause phase separation, almost all of the $^{14}$C-activity was found in the $CHCl_3$ phases (Fig. 2A). When the extract from the 5 min sample of the cerulenin-treated culture was treated in the same manner, however, half of the radioactivity appeared in the $H_2O$-$CH_3OH$ phase (Fig. 2B). The % of the $^{14}$C in the extracts from the cerulenin-treated culture found in the $H_2O$-$CH_3OH$ phase decreased with time so that by 60 min, 96% of the recovered counts was in the $CHCl_3$ phase. Portions of the lipids from the $CHCl_3$ and $H_2O$-$CH_3OH$ phases were used for % desaturation determinations (Fig. 3) while the remainder from each sample was analyzed by thin-layer silica gel chromatography in the presence of unlabeled standards. Fig. 4A shows the radiochromatogram of the $CHCl_3$-phase lipids from the 5 min control sample. The major labeled component (peak 2) co-chromatographed in a variety of solvent systems with authentic phosphatidyl glycerol. The minor component (peak 1), after elution, diazomethane-treatment and further analysis (5) proved to be a mixture of free

*This fatty acid makes up about 65% of the total fatty acids of B. megaterium ATCC 14581 grown at 20°. In a table of a previous publication (3), due to a typographical error, the content of this acid was given as 0.1% while its isomer, 13-methyltetradecanoate, was erroneously listed at 64.6%.

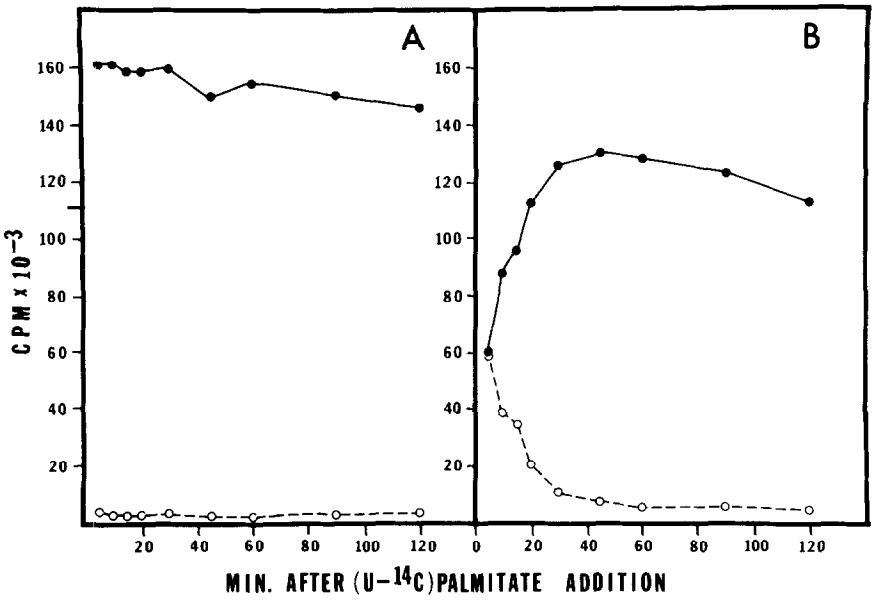
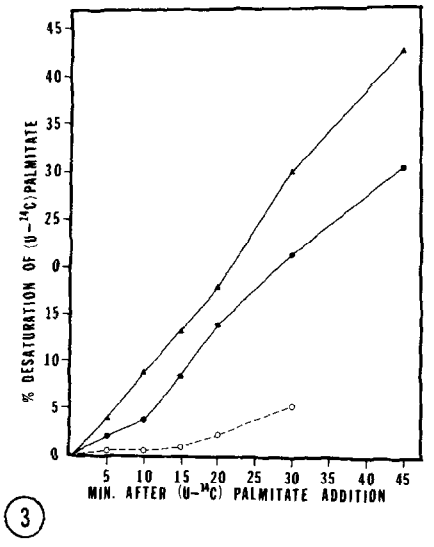



Figure 2. Effects of cerulenin on lipid synthesis from [U-$^{14}$C]palmitate. Two 60 ml portions were removed from a culture growing at 20° when cell density reached 200 Klett units. Portion A served as a control while cerulenin (20 μg/ml) was added to portion B. After 15 min, [U-$^{14}$C]palmitate (0.025 nmoles/ml) was added to each portion, the cells were incubated at 20° and sampled at intervals to determine incorporation of $^{14}$C into lipids. The curves above show the distribution of radioactivity in the $CHCl_3$ phase (•) and the $H_2O$-$CH_3OH$ phase (o) of the extract from each sample. Fig. 2A shows the results from the control culture, Fig. 2B from the cerulenin-treated culture.

fatty acid (65%) and 1,2-diglyceride. Fig. 4B shows a radiochromatogram of the $CHCl_3$-phase lipids from the 5 min cerulenin-treated sample. The $^{14}$C-activity of peak 1 was due to free fatty acid (85-90%) and to 1,2-diglyceride. Peak 2 corresponded to phosphatidyl glycerol while peak X was not immediately identified but was identical to the major radioactive component of the $H_2O$-$CH_3OH$ phase from the same sample (Fig. 4C). Almost all of the radioactivity in the $H_2O$-$CH_3OH$ phase from the 5 min cerulenin-treated sample could be ascribed to this unidentified lipid (lipid X) although a trace of labeled free fatty acid was also found (peak 1, Fig. 4C).



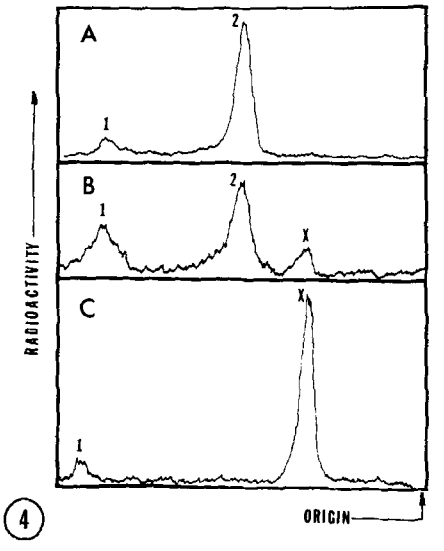




Figure 3. Effect of cerulenin on the desaturation of [U-$^{14}$C]palmitate. The % conversion of labeled palmitate to hexadecenoate was determined for lipid samples obtained during the first 45 min in the experiment described in Fig. 2. The curves (above) show these values for the $CHCl_3$ phases from the control (▲) and the cerulenin-treated (●) cultures and for the $H_2O$-$CH_3OH$ phases of the cerulenin-treated (o) culture. Not enough radioactivity was present in the $H_2O$-$CH_3OH$ phases from the control culture for accurate analysis, nor in the cerulenin-treated culture after 30 min.

Figure 4. Radiochromatogram scans of thin-layer plates used to resolve $CHCl_3$ phase and $H_2O$-$CH_3OH$ phase lipids. Fig. 4A: 5 min control sample, $CHCl_3$ phase. Fig. 4B: 5 min cerulenin-treated sample, $CHCl_3$ phase. Fig. 4C: 5 min cerulenin-treated sample, $H_2O$-$CH_3OH$ phase. Peak identifications include 1, free fatty acid and 1,2-diglyceride; 2, phosphatidyl glycerol; 3, unknown lipid (lipid X). All samples were obtained from the experiment described in Fig. 2.

The lipid composition of the control samples did not change markedly with time. Thus the 2 h sample was almost identical in lipid distribution to the 5 min sample except that a trace ( $< 4\%$) of labeled phosphatidyl ethanolamine was detected. In the extracts from the cerulenin-treated culture, lipid X decreased rapidly with time while phosphatidyl glycerol showed a corresponding increase. There was also an absolute decrease with time in free fatty acid. When the same experiment as described in Fig. 2 was carried out with cultures grown and incubated at 35°, utilization of [U-$^{14}$C]palmitate for lipid synthesis was essentially the same in the control and cerulenin-treated cultures;

both showed rapid incorporation of palmitate into phosphatidyl glycerol but no labeled lipid X was detected, even 5 min after palmitate addition to the cerulenin-treated culture.

Identification of the aqueous methanol-phase component (lipid X) as 1-acyl-lysophosphatidyl glycerol: In a B. megaterium culture at 20° treated with cerulenin and containing $^{32}P$-labeled phosphate, the incorporation of $^{32}P$ into lipid X was readily demonstrated (F.J. Lombardi and A.J. Fulco, unpublished). Lipid X was distinguished from a large variety of other phosphorous-containing lipids on the basis of both chromatographic behavior and specific tests. These included phosphatidic acid, lysophosphatidic acid, cardiolipin, phosphatidyl serine, lysophosphatidyl serine, lysophosphatidyl ethanolamine, phosphatidyl glycerophosphate, CDP-diglyceride, palmityl CoA and palmityl ACP. Lipid X co-chromatographed with unlabeled 1-acyl-lysophosphatidyl glycerol in all solvent systems tested and was also indistinguishable from 1-[U-$^{14}C$] palmityl-lysophosphatidyl glycerol prepared by the action of phospholipase $A_2$ on labeled phosphatidyl glycerol isolated from the control culture in the experiment described under Fig. 2. The $^{14}C$-labeled lysophosphatidyl glycerol thus obtained showed the same solubility properties as lipid X; that is, it entered the aqueous methanol phase when partitioned between $CHCl_3$ and $H_2O$-$CH_3OH$ but was extracted into $CHCl_3$ after acidification of the $H_2O$-$CH_3OH$. When lipid X and $^{14}C$-labeled lysophosphatidyl glycerol, both mixed with unlabeled phosphatidyl glycerol as carrier, were separately subjected to acetolysis (6), identical products resulted. The major radioactive product in each case was diacetyl monopalmityl glycerol derived from displacement of phosphate by acetoxy and by acetylation of the free hydroxy group adjacent to the palmityl moiety. When [U-$^{14}C$]palmitate-labeled phosphatidyl glycerol was subjected to acetolysis under the same conditions, the major radioactive product was monoacetyl diacyl glycerol. Finally, neither lipid X nor authentic 1-acyl-lysophosphatidyl glycerol were affected by phospholipase $A_2$ under conditions

that converted phosphatidyl glycerol to the lyso derivative. Since phospholipase $A_2$ specifically removes only the acyl group in the 2-position of glycerophosphatides (7), we concluded that lipid X was identical to 1-palmitoyl-lysophosphatidyl glycerol.

## DISCUSSION

Although a hypothesis explaining the origin and further metabolism of 1-palmitoyl-lysophosphatidyl glycerol could be derived from results already presented, experiments reported in the following paper lead to more definitive conclusions and these subjects will hence be considered there. It should be noted, however, that the labeled palmitate of lysophosphatidyl glycerol is apparently not available for desaturation while the palmitate incorporated into phosphatidyl glycerol, in the presence or absence of cerulenin, is readily desaturated (Fig. 3). This suggests either that phosphatidyl glycerol (but not its lyso derivative) can be directly utilized as a substrate for desaturation or else that the palmitate of phosphatidyl glycerol rapidly exchanges with hexadecenoate but that the palmitate of lysophosphatidyl glycerol does not. Finally, 1-palmityl-lysophosphatidyl glycerol was formed at 20° but not at 35°. Since we had previously shown that B. megaterium synthesizes unsaturated fatty acids at 20° but not at 35° (3), it seems possible that lysophosphatidyl glycerol may play a specific role in the utilization of newly-formed unsaturated fatty acids for membrane lipid synthesis.

ACKNOWLEDGMENTS: We thank Sue Tsuda for excellent technical assistance in this work and Ellin James for preparation of the figures for publication. These studies were supported in part by Contract EY-76-C-03-0012 between the Environmental Research and Development Administration and the University of California and by Research Grant AI-09829 from the National Institute of Allergy and Infectious Diseases, NIH, USPHS.

## REFERENCES

1. Fujii, D.K. and Fulco, A.J. (1977) J. Biol. Chem. 252, 3660-3670
2. Fulco, A.J. (1972) J. Biol. Chem. 247, 3511-3519
3. Quint, J.F. and Fulco, A.J. (1973) J. Biol. Chem. 248, 6885-6895
4. Omura, S. (1976) Bacteriol. Rev. 40, 681-697
5. Chang, N.C. and Fulco, A.J. (1973) Biochim. Biophys. Acta 296, 287-299
6. Penkonen, O. (1965) J. Am. Oil Chem. Soc. 42, 298-304
7. Shipolini, R., Callewaert, G., Cottrell, R., Doonan, S., Vernon, C. and Banks, B. (1971) Eur. J. Biochem. 20, 459-468