JOURNAL OF BACTERIOLOGY, Oct. 1976, p. 21-27

Vol. 128, No. 1
Printed in U.S.A.

Effect of Cerulenin on the Growth and Differentiation of *Dictyostelium discoideum*

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Received for publication 21 April 1976

The growth of *Dictyostelium discoideum* Ax-2 was inhibited completely by cerulenin at a concentration of 5 μg/ml. This inhibition of growth was found to be due to the inhibition of fatty acid synthesis. Acetate incorporation into a long-chain fatty acid was inhibited completely by cerulenin, and the growth inhibition could be reversed by inclusion of certain saturated fatty acids in the medium. Unsaturated fatty acids and sterols failed to reverse the inhibitory effect. The fatty acid and sterol compositions of cerulenin-treated cells were determined to establish whether the drug could be used to manipulate the organism's lipid composition. Only relatively small manipulations were obtained under the conditions employed in this study. Cerulenin inhibited differentiation but only at high concentrations (150 μg/ml). This inhibition could be reversed by palmitic acid, suggesting that the prime cause of the inhibition was an inhibition of fatty acid synthesis. Thus, it appears that continued fatty acid synthesis is required for the cellular process of differentiation in *D. discoideum*.

The manipulation of membrane lipid composition has proved to be an important method for gaining insight into the role of these molecules in membrane function. Membrane lipid composition has been shown by a large number of workers to markedly alter membrane transport rates and some membrane enzyme activities (2, 8). The modification of membrane fatty acid compositions of a diverse group of organisms, including *Acholeplasma laidlawii* (19), *Acanthamoeba*, and *Tetrahymena* (17), cultured mammalian cells (9, 10) and intact animals (8) has been achieved by the inclusion of fatty acids in the nutrient supply, despite the mitigating influence of endogenous fatty acid synthesis. Although the use of mutants defective in lipid biosynthesis (2, 13, 16) can enhance incorporation, these mutants are extremely difficult to isolate in many organisms, and the method is not generally applicable at the present time.

The antibiotic cerulenin has been shown to restrict the growth of bacteria (11, 25) by inhibiting fatty acid biosynthesis (3, 22). In yeasts, both sterol synthesis and fatty acid synthesis are inhibited by this antibiotic (20, 21). In both bacteria and yeasts growth can be at least partially restored by the addition of the appropriate lipid supplements (11, 20, 25), thus circumventing the necessity for the endogenously synthesized molecules. Thus, cerulenin is potentially a powerful tool for manipulating membrane lipid composition. In this report, we describe the effect of cerulenin on the growth and differentiation of *Dictyostelium discoideum*.

MATERIALS AND METHODS

Materials. Peptone and yeast extract were obtained from Difco. Bovine serum albumin was the fatty acid-poor grade from Calbiochem. All fatty acids and sterols were the best available grade from Sigma Chemical Co., and sodium [^{3}H]acetate and [^{3}H]leucine were obtained from Amersham/Searle. Cerulenin was a gift of S. Ōmura.

Organism and culture conditions. *D. discoideum* Ax-2 was grown at 22°C in a rich nutrient medium containing peptone and yeast extract (Difco), as described previously (23). This medium was supplemented further with glucose to a final concentration of 86 mM and with filter-sterilized bovine serum albumin to a final concentration of 0.4%.

Stock solutions of cerulenin (20 mg/ml in ethanol) were stored at −70°C and diluted to 0.5 mg/ml with water just prior to use. The aqueous solution was filter sterilized, and appropriate samples were added to the growth medium. Fatty acids and sterols were added, where indicated, as sterile ethanolic solutions.

Cell growth was assessed by directly counting cell numbers in a hemocytometer counting chamber. Cell viability was determined by plating dilutions in association with *Enterobacter aerogenes* and counting the clear plaques that formed in the bacterial lawn after incubation at 22°C.

Effect of cerulenin on fatty acid and non-saponifiable lipid synthesis. Cells of *D. discoideum* were grown overnight to a cell density of 1.2×10^6 cells per ml in a medium containing sodium [^{3}H]acetate

at a concentration of 1.2 mM (3 μCi/ml). Varying concentrations of cerulenin were added to different portions of the growing culture, and the cells were incubated for a further 24 h. Fractions (2 ml) were removed, added to 2.0 ml of 15% methanol-potassium hydroxide, and saponified by incubating at 70°C for 1 h. The methanol was removed under a stream of nitrogen, and the volume of the sample was adjusted to approximately 2 ml by the addition of water. The alkaline aqueous solution was extracted three times with 2 ml of pentane to yield the non-saponifiable lipid fraction. The aqueous residue was acidified with 0.3 ml of 24 N H_2SO_4 and reextracted three times with 2 ml of pentane to yield the fatty acid fraction.

The non-saponifiable and fatty acid fractions were dried under nitrogen and redissolved in pentane; this was repeated three times. Each fraction was mixed with toluene scintillation fluid and counted.

Quantitation of cellular protein, fatty acids, and sterols. Cells ($\simeq 5 \times 10^6$/ml) were harvested during the late exponential phase by centrifugation at 700 $\times g$ for 10 min. The cells were then suspended in ice-cold water and recentrifuged for 10 min at 700 $\times g$. This wash procedure was then repeated.

The washed cell pellet was resuspended in ice-cold distilled water, and the protein content of a sample was determined by the Folin procedure (18). The remainder was added to an equal volume of 15% methanolic KOH, and known quantities of cholesterol and arachidic acid were added as internal standards. The mixture was incubated at 70°C for 1 h, and the methanol was then removed under a stream of nitrogen. The remaining aqueous solution was extracted three times with an equal volume of pentane to yield the non-saponifiable fraction. The aqueous residue was then acidified with H_2SO_4 and extracted three more times with equal volumes of pentane to yield the saponifiable fraction.

The non-saponifiable extract was dried under nitrogen, and the residue was suspended in 0.5 ml of 95% ethanol. One milliliter of ethanol, 1.5 ml of acetone, and 1.0 ml of 0.5% digitonin in 80% ethanol were added, and the mixture was incubated overnight at room temperature. The precipitate was removed by centrifugation and washed once with diethyl ether-acetone (2:1 vol/vol) and twice with diethyl ether. The precipitate was dried in a vacuum desiccator, and 0.5 ml of acetic anhydride was added. The suspension was heated to 140°C for 30 min to produce a clear solution. The sterol acetates were dried, suspended in chloroform, and separated by gas-liquid chromatography in a column of 3% SE 30 (isothermally at 245°C; helium gas flow, 80 ml/min). Since digitonin quantitatively precipitates *D. discoideum* sterols and added cholesterol, the amounts of sterol were quantitated by comparison of the peak areas with the peak area of the internal standard cholesterol.

The saponifiable lipid extract was dried under nitrogen, suspended in 1.0 ml of BF_3 in methanol, and incubated at 37°C overnight. Water (1 ml) was added and the mixture was extracted three times with equal volumes of pentane. The pentane extracts were dried under nitrogen, and the methylated fatty acids were separated by gas-liquid chromatography on a column of 12% diethyl glycol-succinate at 150°C. The component fatty acids were identified by comparison with the retention times of authentic standards and by comparison with the previously published fatty acid composition for *D. discoideum* (18). The amount of fatty acid was determined by comparison of the peak areas with the peak areas of the internal standard arachidic acid.

Differentiation of *D. discoideum*. Suspensions of washed cells of *D. discoideum* were spread either onto the surfaces of Whatman no. 50 filter papers soaked in lower pad solution (6) or onto agar surfaces (1).

The cells were incubated at 22°C and observed periodically over the next 36 h.

In experiments in which the effect of cerulenin on differentiation was studied, cells were spread onto the surface of Whatman no. 50 filter papers soaked in lower pad solution containing varying levels of cerulenin. The cells were then incubated at 22°C and observed periodically over the next 72 h.

RESULTS

Effect of cerulenin on the growth of *D. discoideum*. Low concentrations of cerulenin inhibited the growth of *D. discoideum*. In the experiment shown in Fig. 1, growth was inhibited almost totally by 5 μg of cerulenin per ml, inhibited partially by 2 μg/ml and initially slightly inhibited by 1 μg/ml. This dose dependence was slightly variable from experiment to

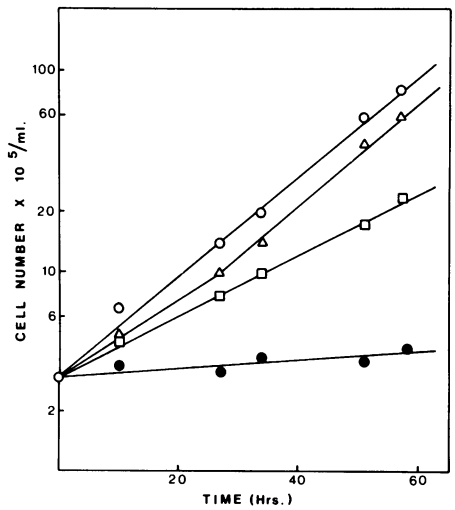

FIG. 1. *Effect of cerulenin on the growth of D. discoideum. Exponentially growing cells of D. discoideum were inoculated into fresh medium containing no cerulenin, ○; 1 μg of cerulenin per ml, □; 2 μg of cerulenin per ml, □; and 5 μg of cerulenin per ml, ●. At the indicated times, portions were removed, and the cell density was assessed by using a microscope with a hemocytometer counting chamber.*

experiment (see Fig. 1 through 4); 5 μg of cerulenin per ml was usually totally inhibitory, but in some experiments slow growth was detectable.

Effect of cerulenin on macromolecular synthesis. The inhibitory effect of cerulenin on the growth of yeasts was found to be due to the inhibition of fatty acid and sterol synthesis (13, 14). In *D. discoideum*, fatty acid synthesis was inhibited partially at 2 μg/ml and totally inhibited by cerulenin concentrations of 5 μg/ml or greater (Table 1). Furthermore, there was no increase in the incorporation of radioactivity into the fatty acids after the addition of 5 μg of cerulenin per ml, suggesting that the inhibition is immediate. In contrast, the incorporation of acetate into the non-saponifiable lipid was only partially inhibited at cerulenin concentrations as high as 50 μg/ml (Table 1). These results suggest that the growth inhibition of *D. discoideum* is due primarily to the absence of fatty acid synthesis, although the inhibition of non-saponifiable lipid synthesis might also be a contributing factor.

Reversal of growth inhibition of cerulenin by certain exogenous fatty acids. If the inhibitory effect of cerulenin on the growth of *D. discoideum* was, in fact, due to a specific inhibition of lipid synthesis, then the addition of exogenous lipids to the growth medium should reverse this inhibition. Such a reversal was observed with certain fatty acids (Fig. 2). Two hundred micromolar concentrations of either palmitic acid or myristic acid almost completely restored the rate of growth of *D. discoideum* to that of control cells incubated in the absence of cerulenin. The final cell density attained, however, was only about 60% of that of the control cultures. Stearic acid (200 μM), nonadecanoic acid (200 μM), and heptadecanoic acid (200 μM) also partially reversed the inhibition by cerulenin but were less effective than either palmitic acid or myristic acid (Fig. 2). Arachidic acid (200 μM) allowed only poor growth in the presence of cerulenin.

TABLE 1. *Effect of cerulenin on fatty acid and non-saponifiable lipid synthesis*

Addition to growth medium (μg/ml)	Time after cerulenin addition (h)	Incorporation of [^{3}H]acetate[a] (cpm × 10^3)	
		Non-saponifiable lipid	Fatty acid
No cerulenin	0	1.6	70.3
No cerulenin	14	6.0	335.4
Cerulenin			
2	14	4.6	78.5
5	14	2.7	66.2
20	14	2.4	65.7
50	14	2.6	68.9
No cerulenin	24	9.2	620.2
Cerulenin			
2	24	5.8	165.8
5	24	4.8	65.9
20	24	3.7	67.2
50	24	3.4	67.1

[a] Each value represents the counts per minute incorporated per 2-ml volume of cell culture and is the mean of duplicate determinations.

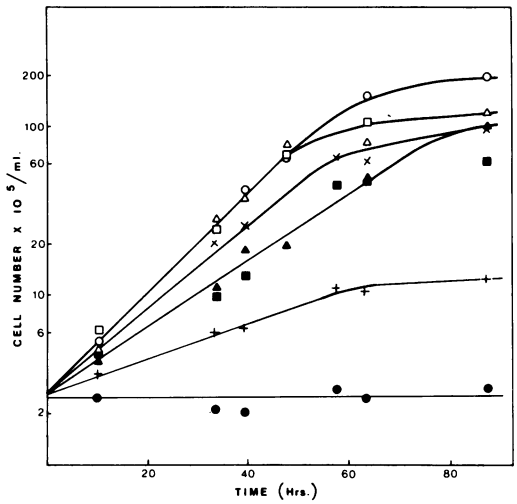

FIG. 2. *Effect of exogenous saturated fatty acids on the growth of D. discoideum in the presence of cerulenin. Exponentially growing cells of D. discoideum were inoculated into a number of flasks, each containing fresh medium plus 5 μg of cerulenin per ml. In addition, each of the flasks was supplemented with one of the following fatty acids: 200 μM palmitic, △; 200 μM myristic, □; 200 μM stearic, ×; 200 μM arachidic, +; 200 μM heptadecanoic, ▲; 200 μM nonadecanoic, ■; no fatty acid, ●. Control media contained no cerulenin and no fatty acid, ○. At the indicated times, portions of cell suspensions were removed from the growth flasks, and the cell density was assessed by using a microscope with a hemocytometer counting chamber.*

In contrast, monoenoic fatty acids failed to significantly reverse the inhibitory effects of cerulenin (Fig. 3). Only oleic acid (200 μM) supported more than a doubling of the cell number. Similarly, it was found that polyenoic fatty acids at concentrations of 200 μM only slightly reversed the inhibitory effects of cerulenin (Fig. 4). In this experiment, cerulenin (5 μg/ml) allowed slight growth of *D. discoideum*, but none of the polyenoic fatty acids produced more than a doubling of the cell density above this low background growth. The unsaturated fatty acids (Fig. 3 and 4) were far less effective than any of the saturated fatty acids (Fig. 2) in

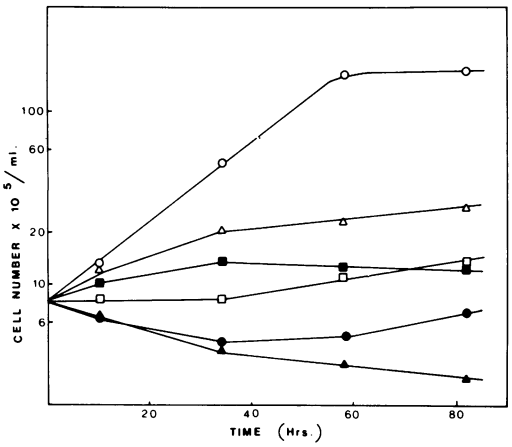

FIG. 3. *Effect of monounsaturated fatty acids on the growth inhibition of D. discoideum by cerulenin. Experimental details as stated in the legend of Fig. 2, except that the supplemented fatty acids were: 200 μM oleic acid, △; 200 μM cis-vaccinic acid, □; 200 μM elaidic acid, ■; 200 μM palmitoleic acid, ▲; no fatty acid, ●. Controls contained no cerulenin and no fatty acid, ○.*

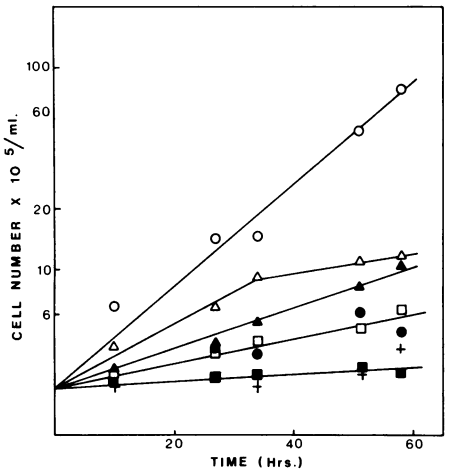

FIG. 4. *Effect of polyunsaturated fatty acids and sterols on the growth inhibition of D. discoideum by cerulenin. Experimental details as stated in the legend of Fig. 2, except that the supplemented fatty acids or sterols were: 200 μM linoleic acid, □; 200 μM linolenic acid, △; 200 μM arachidonic acid, ▲; 200 μM cholesterol, ■; 200 μM stigmasterol, +; no supplementation, ●. Controls contained no cerulenin and no supplementation, ○.*

reversing the inhibitory effects of the cerulenin. This is not due to an independent inhibitory effect of the unsaturated fatty acid themselves, since they do not inhibit growth under the conditions employed in the present study (G. Weeks, Biochim. Biophys. Acta, in press). Neither cholesterol nor stigmasterol at a concentration of 200 μM was able to reverse the inhibitory effects of cerulenin (5 μg/ml), a result consistent with the observations that the most pronounced inhibitory effect of the antibiotic was on fatty acid synthesis.

The inhibition of growth by cerulenin was reversed almost completely by either palmitic acid (200 μM) or myristic acid (200 μM). However, this reversal was markedly dependent upon the concentration of the supplemented fatty acid. Lower concentrations of palmitic acid produced proportionally less reversal of the growth inhibition, as shown in Table 2. Both the growth rate and the extent of growth were influenced markedly by the concentration of the supplemented palmitic acid. Also shown in Table 2 are the results of a similar experiment, using a lower concentration of cerulenin (2 μg/ml). Growth inhibition at this concentration of the antibiotic was only partial (as in Fig. 1), and the reversal of inhibition was much more pronounced at all concentrations of palmitic acid, both growth rate and extent of growth being fully restored by 200 μM palmitic acid.

Effect of cerulenin on the viability and differentiation of *D. discoideum*. Although short exposures of cells to cerulenin had no effect on their viability, a 48-h exposure to cerulenin at 5 μg/ml reduced cell viability by 75%. Differentiation of the surviving viable cells was normal. Cells grown in the presence of cerulenin and the saturated fatty acids also exhibited normal differentiation.

When cells grown in the absence of cerulenin were spread on filter papers in the presence of cerulenin at a concentration of 150 μg/ml, the differentiation process was completely in-

TABLE 2. *Reversal of cerulenin-inhibited growth by different concentrations of palmitic acid*[a]

Addition to growth medium (μM)	Cerulenin, 2 μg/ml		Cerulenin, 5 μg/ml	
	Generation time (h)	Cell density attained (cells/ml)	Generation time (h)	Cell density attained (cells/ml)
No palmitic acid	42	2.0×10^6	NG[b]	4.6×10^5
Palmitic acid				
25	25.5	3.1×10^6	105	6.4×10^5
50	17.5	6.2×10^6	22	9.2×10^5
75	16	8.3×10^6	22	2.1×10^6
100	12.5	1.7×10^7	15	2.8×10^6
200	10	2.1×10^7	10	6.6×10^6

[a] Controls containing no cerulenin and no fatty acid supplement grew to a density of 2.1×10^7 cells per ml with a generation time of 10 h.

[b] NG, No growth.

hibited, such that there was no sign of aggregate formation even after 48 h of incubation. The inhibition was reversed completely if cells were incubated in the presence of palmitic acid (200 μM) in addition to the cerulenin. Differentiation was normal for cells incubated in the presence of 100 or less μg of cerulenin per ml.

Effect of cerulenin on the fatty acid and sterol compositions of *D. discoideum*. Cerulenin (5 μg/ml)-treated cells were grown in the presence of the saturated fatty acids that promoted significant growth. The cells were harvested during the exponential phase of growth, and the sterol and fatty acid compositions were determined. Cells grown in a medium with cerulenin plus exogenous fatty acid contained less fatty acid per milligram of protein than control cells (Table 3), suggesting that the exogenously supplied fatty acid did not completely replace the normal level of endogenously synthesized fatty acid. The sterol content of cells grown in the presence of cerulenin plus fatty acid was also lower than that of control cells (Table 3), probably reflecting the reduced rates of nonsaponifiable lipid synthesis observed in the presence of cerulenin.

TABLE 3. *Sterol and fatty acid content of D. discoideum grown medium containing cerulenin and fatty acid*

Addition to medium	Content		Sterol/fatty acid ratio
	Fatty acid (μg/mg of protein)	Sterol (μg/mg of protein)	
No cerulenin, no fatty acid	55.2	14.5	0.26
Cerulenin plus[a]:			
Palmitic acid	41.4	9.2	0.22
Stearic acid	35.1	7.9	0.22
Heptadecanoic acid	35.5	8.7	0.24
Nonadecanoic acid	44.1	7.9	0.18

[a] Cerulenin, 5 μg/ml; acids, 200 μM concentration each.

The fatty acid composition of cells grown in the presence of cerulenin plus added fatty acids is shown in Table 4. Cerulenin-treated cells supplemented with palmitic acid had a fatty acid composition almost identical to that of control cells grown in the absence of cerulenin. Cells supplemented with stearic acid had a slightly altered fatty acid composition, with elevated levels of stearic acid and slightly reduced levels of palmitic acid. In contrast, cells supplemented with heptadecanoic acid or nonadecanoic acid had markedly altered fatty acid compositions. In both cases, between 65 and 70% of the total cellular fatty acid possessed an odd number of carbon atoms and were almost certainly derived from the medium. It would appear that the C_{17} acids are elongated to C_{19} acids when cells are grown in the presence of heptadecanoic acid and that the C_{19} acids are oxidized to C_{17} fatty acids when cells are supplemented with nonadecanoic acid.

The sterol compositions of cells grown under these various conditions were very similar (data not shown). In all instances, over 80% of the sterols had a retention time characteristic of Δ^{22}-stigmasten-3-β-ol, shown previously to be the major sterol in *D. discoideum* (12, 15). In addition, approximately 5% of the sterol content had a retention time characteristic of stigasmanol, which has been shown to be a minor sterol constituent in *D. discoideum* (7). The remaining sterol component, 5 to 10% of the total, has yet to be identified.

TABLE 4. *Fatty acid composition of D. discoideum grown in the presence of cerulenin and various saturated fatty acids*

Addition to medium	Fatty acid composition (%)															Total saturated fatty acids[a]
	14:0	15:0	Palmitaldehyde	16:0	16:1	16:2 17:0[b]	17:1	17:2	18:0	18:1	18:2	19:0	19:1	19:2	Other[c]	
No cerulenin, no fatty acid	0.9		3.6	6.8	4.0	3.5	0.3	0.8	1.2	34.2	40.6				4.1	12.5
Cerulenin plus[d]:																
Palmitic acid	1.3		1.9	7.0	3.5	2.3	0.4	0.8	2.6	29.6	44.0				6.7	12.8
Stearic acid	0.4		4.5	5.1	2.0	0.7	0.6	0.4	6.1	28.8	45.8				5.5	16.1
Heptadecanoic acid	2.2	2.2	0.5	4.9	0.6	7.2	7.2	21.3	2.5	8.0	9.1	14.0	9.9	6.4	4.0	26.3
Nonadecanoic acid	0.5	0.5	0.3	2.5	0.6	3.5	5.6	17.2	0.7	7.0	13.7	26.1	10.8	7.2	3.7	30.1

[a] Sum of all of the saturated fatty acids plus palmitaldehyde.
[b] Not separated under the conditions used in this study, but both were shown to be present by Davidoff and Korn (4).
[c] Several minor unidentified fatty acids.
[d] Cerulenin, 5 μg/ml; acids, 200 μM concentration each.

DISCUSSION

The growth of *D. discoideum* was found to be completely inhibited by the antibiotic cerulenin at a concentration of 5 μg/ml. Thus, the organism is less sensitive to the antibiotic than the yeast *Candida stellatoida* (20) but more sensitive than the bacteria *Bacillus subtilis* and *Escherichia coli* (11). In the yeast, both fatty acid synthesis and sterol synthesis were found to be inhibited by cerulenin, and growth was restored by the addition of either a fatty acid or a sterol (20, 21); in bacteria, only fatty acid synthesis is inhibited, and growth can be restored by the addition of the appropriate combination of long-chain fatty acids (11, 25). In *D. discoideum*, only fatty acid synthesis is inhibited markedly; even at high concentrations of the antibiotic, the synthesis of non-saponifiable lipids is only reduced partially. Consequently, the growth of cerulenin-treated cells was almost completely restored by the addition of certain exogenous fatty acids but not by the addition of cholesterol or stigmasterol.

Cerulenin at a high concentration (150 μg/ml) completely inhibited the differentiation of *D. discoideum*. Furthermore, this inhibition was reversed by the addition of 200 μM palmitic acid. Thus, continued lipid synthesis may be necessary for the cellular processes involved in the differentiation of this organism. It is noteworthy that much higher concentrations of cerulenin are necessary for the inhibition of differentiation compared with growth. Earlier studies showed that growth is inhibited at much lower concentrations of cyclohexamide and actinomycin D than is aggregation (14), and it is possible that the non-proliferating cells may be less permeable to the antibiotics. Of the fatty acids tested, only palmitic acid and myristic acid restored growth to the control rate. Furthermore, the fatty acid composition of cells grown in the presence of cerulenin and palmitic acid was nearly identical to that of untreated controls, suggesting that all of the major fatty acids of *D. discoideum* can be synthesized from palmitic acid. This not only is consistent with the biosynthetic pathway proposed by Davidoff and Korn (4), but also shows that the inhibition of de novo fatty acid synthesis by cerulenin does not extend to the fatty acyl chain elongation system of *D. discoideum*. Although the reason for this remains obscure, the insensitivity of in vitro chain elongation to cerulenin has been demonstrated in *Mycobacterium phlei* by Vance et al. (22). It should be noted that the growth yield of cerulenin-treated cells supplemented with palmitic acid or myristic acid is not as great as that of controls. Perhaps there is a minor fatty acid component that cannot be synthesized from either of these supplements, or, alternatively, some component(s) of the partially inhibited, non-saponifiable lipid fraction might be required for maximal yields. The ratio of fatty acid to milligrams of protein was slightly lower in cerulenin-fatty acid-grown cells than in controls, which may also have some bearing on the lowered growth yield.

Stearic acid only partially restored the growth of cerulenin-treated *D. discoideum*, which may reflect the inefficiency with which this acid can be used as precursor of other saturated or unsaturated fatty acids. Heptadecanoic acid and nonadecanoic acid also partially restored cell growth of cerulenin-inhibited cells. In this case, the fatty acid composition of the cells was markedly different from that of the controls. Substitution of the normal fatty acids by fatty acids of an odd chain length might slightly impair some cellular functions and result in the observed slow rate of growth. Furthermore, these cells contain considerably elevated levels of saturated fatty acids, which may also result in impaired cellular function.

Arachidic acid and all of the unsaturated fatty acids tested allowed little or no growth in the presence of cerulenin. Arachidic acid is not desaturated by *D. discoideum* (Weeks, in press), and its rate of beta oxidation to stearic acid or palmitic acid may be sufficient for only slow rates of growth. The absence of growth in the presence of the monounsaturated fatty acids is interesting, because in *D. discoideum* almost 90% of the total fatty acids are unsaturated. This may indicate that the small levels of saturated fatty acid can not be synthesized from the supplied unsaturated fatty acid and are absolutely essential for growth.

It was hoped at the outset of this work that the use of cerulenin might allow manipulation of the sterol composition of the organism. However, since sterol synthesis is only partially inhibited by cerulenin, dramatic modification of the sterol content may prove to be impossible using this approach. The sterol content per milligram of protein was reduced (Table 3), but the effect on cellular growth was minimal.

All cells grown in the presence of cerulenin and fatty acid differentiated normally even though in some cases they grew at reduced growth rates. Thus the modification of cellular lipid observed in these experiments had no adverse effect on the cell-cell interaction that accompanies differentiation in this organism.

ACKNOWLEDGMENTS

We wish to thank S. Ōmura for kindly supplying the cerulenin, without which this work would have been impos-

sible. We also wish to thank C. Weeks for critically reading the manuscript.

This research was supported by a grant from the National Research Council of Canada.

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