INHIBITION OF STEROL AND FATTY ACID BIOSYNTHESES BY CERULENIN IN CELL-FREE SYSTEMS OF YEAST

Sir:

Cerulenin is an antifungal antibiotic isolated from the culture filtrate of *Cephalosporium caerulens*[1~4]. The antibiotic strongly inhibited the growth of yeast-type fungi, and moderately inhibited the growth of filamentous fungi *in vitro*[5]. The chemical structure of cerulenin was reported as (2S) (3R) 2, 3-epoxy-4-oxo-6, 10-dodecadienoylamide[6,7]. Cerulenin was found to cause a curling of the mycelium of filamentous fungi such as *Trichophyton* and *Microsporium*[8~10].

The action mechanism of cerulenin has been investigated with *Candida stellatoidea* (*C. stellatoidea*) and *Saccharomyces cerevisiae* (*S. cerevisiae*)[11]. In the previous experiments using the whole cells of *C. stellatoidea*, it has been observed to inhibit neither the biosyntheses of nucleic acids, protein, and cell wall, nor the exogenous respiration of the yeast. However, the antifungal activity of cerulenin was reversed especially by ergocalciferol, and to a certain extent by lauric acid and oleic acid. The amount of intracellular ergosterol of the yeast was decreased by cerulenin.

Moreover, the incorporation of 1-^{14}C-acetate into nonsaponifiable fraction (NSF) and fatty acid fraction (FAF) was inhibited at low concentrations of cerulenin. From these experimental results, it was suggested that cerulenin acts on the biosyntheses of sterols and fatty acids in yeast.

The action mechanism of cerulenin was further investigated with the cell-free systems of *S. cerevisiae*. These experimental results are presented in this communication.

S. cerevisiae ATCC 12341 was grown semi-anaerobically at 27°C for 48 hours in a medium containing 2 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 0.5 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.0025 g of $MnSO_4 \cdot 4H_2O$, 20 g of glucose and 1 g of yeast extract per liter according to the methods of KATSUKI-BLOCH[12] and KAWAGUCHI[13].

Then, 1.5% of the culture broth was inoculated in the same medium and incubated semi-anaerobically at 27°C for 24 hours. Cells were harvested, washed twice with 0.1 M potassium-sodium phosphate buffer (pH 6.2), suspended in the same buffer containing 10% of glucose, and shaken at 27°C for 150 minutes according to the method of KLEIN[14].

The cells were reharvested by centrifugation, and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). The cells (60 g of wet weight) were resuspended in 60 ml of the same buffer and disrupted with 60 g of glass beads by a Braum cell homogenizer (type MSK). The cell homogenate was centrifuged at $5,000 \times g$ for 1 hour, and the supernatant was recentrifuged at $80,000 \times g$ for 1 hour. The supernatant (60 ml), discarding the floating lipid layer, was used as the cell-free extract. Reaction mixture, incubation condition and isolation of FAF and NSF are described in the Figures and the Table.

The effects of cerulenin on the incorporation of 1-^{14}C-acetate into FAF and NSF, and of 2-^{14}C-mevalonate into NSF were studied in the cell-free systems of *S. cerevisiae*. These results are shown in Fig. 1.

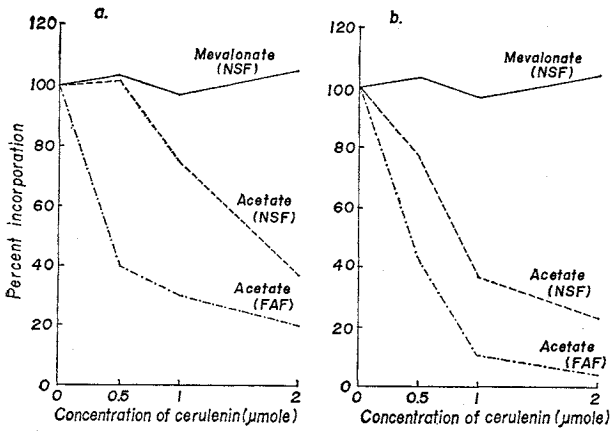
The incorporation of acetate into FAF and NSF were inhibited remarkably by cerulenin, but that of mevalonate into NSF was not inhibited. In addition, the inhibition of acetate incorporation into FAF and NSF varied with the degree of incorporation of this labeled substrate into each fraction. When the incorporation of acetate into FAF was the same degree as that into NSF, the incorporations into FAF and NSF were inhibited by 80% and 63%, respectively, with 2 μmoles of cerulenin (Fig. 1-a). However, when the incorporation of acetate into FAF was higher than that into NSF, the incorporations of acetate into FAF and NSF were inhibited by 96% and 77%, respectively, by this antibiotic (Fig. 1-b). In both cases, cerulenin inhibited more strongly the incorporation of labeled acetate into FAF than that into NSF.

These facts coincide with the experimental results in the whole cells of yeast, and it was proved that cerulenin acts on the biosyntheses of sterols and fatty acids in yeast. Morerover, since cerulenin did not inhibit

Fig. 1. Effect of cerulenin on the incorporation of 1-^{14}C-acetate into FAF and NSF, and of 2-^{14}C-mevalonate into NSF in the cell-free systems of *S. cerevisiae*.

(a) Effect of cerulenin on the incorporation of acetate into FAF when the incorporation was the same degree as that into NSF

(b) Effect of cerulenin on the incorporation of acetate into FAF when the incorporation was the higher than that into NSF.



Reaction mixture contained the following components, in a final volume of 1.0 ml: ATP, 5 μmoles; CoA, 0.1 μmole; NADP, 1 μmole; GSH, 2 μmoles; glucose-6-phosphate, 10 μmoles; $MgSO_4 \cdot 7H_2O$, 2 μmoles; $MnSO_4 \cdot 4H_2O$, 1 μmole; phosphate buffer (pH 7.0), 80 μmoles; 0.35 ml of cell-free extract (20 mg of protein) and cerulenin.

The reaction mixture was incubated at 37℃ for 15 minutes with shaking. After the preincubation, 1-^{14}C-sodium acetate (49.3 μCi/μM), 0.002 μmole (440,000 cpm) or 2-^{14}C-potassium mevalonate (5.85 μCi/μM), 0.0171 μmole (44,000 cpm) was added to the mixture, and further incubated at 37℃ for 1 hour.

The reaction mixture was saponified with 15% KOH in 80% methanol at 60℃ for 1 hour. FAF and NSF were isolated with petroleum ether. The radioactivity was measured in a gas-flow counter.

Fig. 2. Effect of avidin on the incorporation of 1-^{14}C-acetate into FAF and NSF in the cell-free system of *S. cerevisiae*.

Curve I: Effect of avidin on the incorporation of acetate into NSF.

Curve II: Effect of avidin on the incorporation of acetate into FAF when the incorporation was the same degree as that into NSF.

Curve III: Effect of avidin on the incorporation of acetate into FAF when the incorporation was higher than that into NSF.

The reaction mixture except the addition of ^{14}C-mevalonate and cerulenin, the incubation condition and the isolation of FAF and NSF were the same as those in Fig. 1.

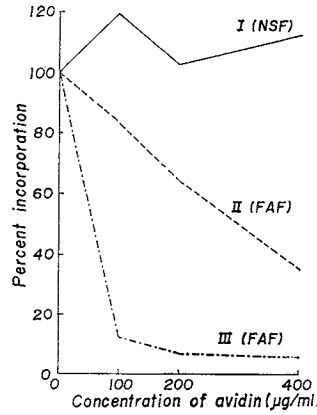


Table 1. Inhibition by cerulenin on the incorporation of ^{14}C-labeled compounds into FAF and NSF in the cell-free systems of *S. cerevisiae*

Radioactive substrate	Dose of cerulenin (μmole)	FAF		NSF	
		Incorporation (cpm)	Inhibition (%)	Incorporation (cpm)	Inhibition (%)
1-^{14}C-Acetate	0	75,730	—	35,978	—
	2.0	21,448	71.7	11,810	67.2
	3.0	10,184	86.6	5,429	84.9
1-^{14}C-Acetyl-CoA	0	7,011	—	2,555	—
	2.0	2,362	66.3	1,681	34.2
	3.0	1,038	85.2	1,090	57.3
1,3-^{14}C-Malonyl-CoA	0	19,644	—		
	2.0	1,897	90.3		
	3.0	1,081	94.5		

The reaction mixture except the addition of ^{14}C-mevalonate, the incubation condition and the isolation of FAF and NSF were the same as those in Fig. 1.

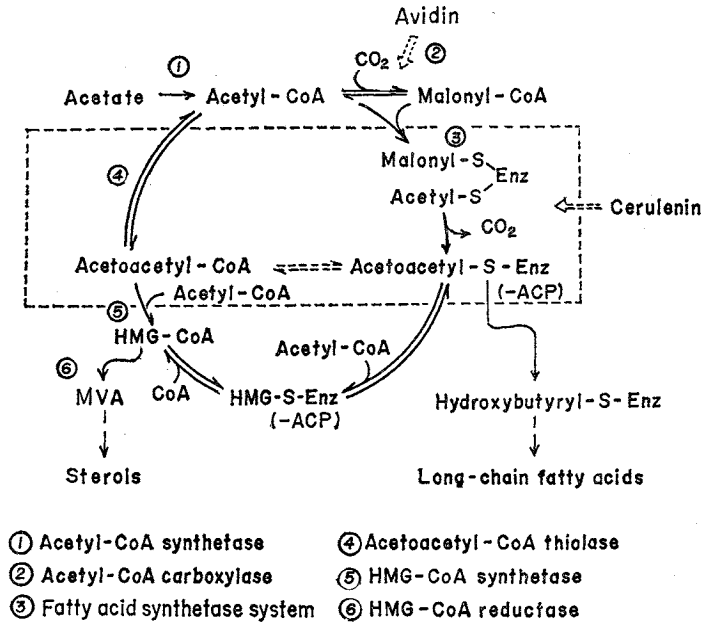
1-^{14}C-Acetyl-CoA (48.6 μCi/μM), 0.0021 μmole (88,000 cpm) or 1,3-^{14}C-malonyl-CoA (19.8 μCi/μM), 0.0051 μmole (88,000 cpm) was added to the reaction mixture.

In the experiment on the incorporation of ^{14}C-malonyl-CoA, 0.002 μmole of unlabeled acetyl-CoA was added to the reaction mixture as a substrate.

the incorporation of mevalonate into NSF, it was suggested that cerulenin inhibits a early step of the pathway between acetate and mevalonic acid (MVA) in sterol and fatty acid biosyntheses.

Then, the action of avidin which inhibits the reaction of acetyl-CoA carboxylase in yeast[15,16] was investigated and compared with the action of cerulenin in the cell-free system of *S. cerevisiae*. As the result, the incorporation of ^{14}C-acetate into FAF was inhibited by 80% at the concentration of 100 μg/ml of avidin, but that into NSF was not affected or rather stimulated by this inhibitor. In addition, avidin inhibited strongly the incorporation of acetate into FAF when the incorporation was higher than that into NSF. These results are shown in Fig. 2.

Scheme 1.



From these results, it was suggested that the action of cerulenin is different from that of avidin, and cerulenin does not act on the step of malonyl-CoA formation. Furthermore, these experimental results indicate the existence of another pathway which does not pass through malonyl-CoA on sterol biosynthesis in yeast.

Next, the action of cerulenin was investigated on the incorporation of ^{14}C-labeled compounds into FAF and NSF. The incorporations of 1-^{14}C-acetate, 1-^{14}C-acetyl-CoA and 1,3-^{14}C-malonyl-CoA into FAF were inhibited by 2.0 and 3.0 μmoles of cerulenin by 72, 87%; 66, 85%; and 90, 95%, respectively. In addition, the incorporation of 1-^{14}C-acetate and 1-^{14}C-acetyl-CoA into NSF were inhibited by 3.0 μmoles of the antibiotic by 85% and 57%, respectively. The inhibition by cerulenin on the incorporation of these substrates into FAF was stronger than that into NSF. These results are summarized in Table 1.

RUDNEY and coworkers[17] reported that there are two pathways in the formation of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) or MVA in yeast. One pathway involves the formation of acetoacetyl-CoA from acetyl-CoA catalized by β-ketoacyl-CoA thiolase, and subsequent condensation of this CoA ester with acetyl-CoA to form HMG-CoA. The other one, the so-called malonyl-CoA pathway, involves the formation of malonyl-CoA from acetyl-CoA and the conversion to acetoacetyl-ACP (acyl-carrier-protein). They suggested that enzyme bound HMG-ACP is an intermediate in HMG-CoA formation when acetoacetyl-ACP is the substrate.

On the other hand, fatty acid synthetase system in yeast was discovered as a multienzyme complex, and the principal hypothesis of fatty acid biosynthesis was proposed by LYNEN and coworkers[18~21]. According to the hypothesis, the formation of malonyl-CoA catalized by acetyl-CoA carboxylase is a primary step in the biosynthesis, and the long-chain fatty acids are formed by the synthetase system from malonyl-CoA and acetyl-CoA. The early pathways of sterol and fatty acid biosyntheses are shown in Scheme 1.

From these biochemical information and the above experimental results, it is suggested that cerulenin acts on the step after

malonyl-CoA formation in the early pathway on fatty acid biosynthesis involved MVA formation on sterol biosynthesis.

Consequently, it is probable that cerulenin inhibits the condensation reaction of acyl thioester (acetyl-S-enzyme) and malonyl thioester (malonyl-S-enzyme) to form β-ketoacyl-S-enzyme (acetoacetyl-S-enzyme) on the fatty acid synthetase in yeast.

Moreover, since it is considered that cerulenin may act on the formation of acetoacetyl-CoA catalized by β-ketoacyl-CoA thiolase, further investigations on the mechanism of action of cerulenin are now progress in details.

The authors wish to express their sincere thanks to Dr. A. KAWAGUCHI, Intstitute of Applied Microbiology, University of Tokyo, for his valuable guidance and his generous gift of the strain of *S. cerevisiae*.

SETSUZŌ NOMURA
TADASHI HORIUCHI
TOJU HATA

Kitasato Institute and
Kitasato University,
Minato-ku, Tokyo, Japan

SATOSHI ŌMURA

Wesleyan University,
Middletown, Connecticut,
U.S.A.

(Received March 6, 1972)

References

1) HATA, T.; Y. SANO, A. MATSUMAE, Y. KAMIO, S. NOMURA & S. SUGAWARA: Studies on a new anticandida substance. Abstr. of the 33 rd Annual Meeting of the Japan Bacteriological Society. Japan. J. Bact. 15 : 1075～1076, 1960
2) MATSUMAE, A.; Y. KAMIO & T. HATA: Studies on cerulenin. I. Studies on cerulenin producing strain. J. Antibiotics, Ser. A 16 : 236～238, 1963
3) MATSUMAE, A.; S. NOMURA & T. HATA: Studies on cerulenin. II. Biological assay and fermentation of cerulenin. J. Antibiotics, Ser. A 16 : 239～243, 1963
4) SANO, Y.; S. NOMURA, Y. KAMIO, S. ŌMURA & T. HATA: Studies on cerulenin. III. Isolation and physico-chemical properties of cerulenin. J. Antibiotics, Ser. A 20 : 344～348, 1967
5) MATSUMAE, A.; S. NOMURA & T. HATA: Studies on cerulenin. IV. Biological characteristics of cerulenin. J. Antibiotics, Ser. A 17 : 1～7, 1964
6) ŌMURA, S.; M. KATAGIRI, A. NAKAGAWA, Y. SANO, S. NOMURA & T. HATA: Studies on cerulenin. V. Structure of cerulenin. J. Antibiotics, Ser. A 20 : 349～354, 1967
7) ŌMURA, S.; A. NAKAGAWA, K. SEKIKAWA, M. OTANI & T. HATA: Studies on cerulenin. VI. Some spectroscopic features of cerulenin. Chem. Pharm. Bull. 17 : 2361～2363, 1969
8) HATA, T.; A. MATSUMAE, S. NOMURA, T. KIM & K. RYAN: Studies on cerulenin, a new antifungal antibiotic. II. Biological characteristics and therapeutic effect of cerulenin. Abstr. of the 4 th Annual Meeting of the Japanese Society for Medical Mycology. Japan. J. Med. Mycol. 1 : 382～383, 1960.
9) HATA, T.; A. MATSUMAE & K. RYAN: Studies on the antifungal activity of cerulenin. Abstr. of the 34 th Annual Meeting of the Japan Bacteriological Society. Japan. J. Bacteriol. 16 : 743～744, 1961
10) IWATA, K.; K. YOKOTA, A. MATSUDA, S. YOSHIKAWA & K. WAKABAYASHI: Studies on the mode of action of antifungal substances. Japan. J. Med. Mycol. 3 : 85～104, 1962
11) NOMURA, S.; T. HORIUCHI, S. ŌMURA & T. HATA: The action mechanism of cerulenin. I. Effect of cerulenin on sterol and fatty acid biosyntheses in yeast. J. Biochem. 71 : 783～796, 1972
12) KATSUKI, H. & K. BLOCH: Studies on the biosynthesis of ergosterol in yeast. Formation of methylated intermediates. J. Biol. Chem. 242 : 222～227, 1967
13) KAWAGUCHI, A.: Control of ergosterol biosynthesis in yeast. Existence of lipid inhibitors. J. Biochem. 67 : 219～227, 1970
14) KLEIN, H. P.: Synthesis of lipids in resting cells of *Saccharomyces cerevisiae*. J. Bact. 69 : 620～627, 1955
15) MATSUHASHI, M.; S. MATSUHASHI, S. NUMA & F. LYNEN: Zur Biosynthese der Fettsäuren. IV. Acetyl-CoA Carboxylase aus Hefe. Biochem. Zts. 340 : 243～262, 1964
16) MATSUHASHI, M.; S. MATSUHASHI & F. LYNEN: Zur Biosynthese der Fettsäuren. V. Die Acetyl-CoA Carboxylase aus Rattenleber und ihre Aktivierung durch Citronensäure. Biochem. Zts. 340 : 263～289, 1964
17) RUDNEY, H.; P. R. STEWART, P. W. MAJERUS & P. R. VAGELOS: The biosynthesis of β-hydroxy-β-methylglutaryl coenzyme A in yeast. V. The role of acyl carrier protein. J. Biol. Chem. 241 : 1226～1228, 1966
18) LYNEN, F.: Biosynthesis of saturated fatty acids. Fed. Proc. 20 : 941～951, 1961
19) LYNEN, F.; I. HOPPER-KESSEL & H. EGGERER: Zur Biosynthese der Fettsäuren. III. Die Fattsäurensynthetase der Hefe und die Bildung enzymgebundener Acetessigsäure. Biochem. Zts. 340 : 95～124, 1964
20) LYNEN, F.: The role of biotin-dependent carboxylations in biosynthetic reactions. Biochem. J. 102 : 381～400, 1967
21) LYNEN, F.; A. HAGEN, I. HOPPER-KESSEL, D. OESTERHECT, H. C. REEVES, E. SCHWEIZER, K. WILLECKE & M. YALPANI: Multienzyme complex of fatty acid synthetase. "Organizational Biosynthesis", ed. by H J. VOGEL, J. O. LAMPEN & V. BRYSON, Acad. Press, New York, pp. 243～266, 1967