ON THE MODE OF ACTION OF CRYOMYCIN

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The mode of action of cryomycin upon *Staphylococcus aureus* IFO 3061 and *Bacillus subtilis* IFO 3037 has been investigated. Cryomycin inhibited the syntheses of protein, RNA and DNA to almost the same degree. It caused a rapid increase of endogenous and exogenous respiration in the test organisms at minimum growth inhibitory concentrations. It was concluded that the primary effect of cryomycin was on the coupling of oxidative phosphorylation followed by secondary effects such as membrane damage, and protein and nucleic acid syntheses. Cryomycin also caused the leakage of $A_{260\,m\mu}$ absorbing materials from most of the *Bacillus* species.

Cryomycin is a new peptide antibiotic isolated in our laboratory. It is produced only at low temperatures by *Streptomyces griseus* subsp. *psychrophilus* AKU 2881. Its production, isolation and properties were described in preceding papers.[1,2] Cryomycin strongly inhibits the growth of Gram-positive bacteria, and less effectively the growth of Gram-negative bacteria, yeasts and fungi.

Since cryomycin is a unique substance isolated from a psychrophilic *Streptomyces*, its mode of action has been studied using sensitive microorganisms. In this paper, we wish to report that the primary effect of cryomycin is on respiration.

Materials and Methods

1. Strains: *Bacillus subtilis* IFO 3037 and *Staphylococcus aureus* IFO 3061 were used.

2. Culture medium: The following nutrient medium was used for the growth of test organisms. Thirty g Nissan Dry Bouillon (Nissui Seiyaku Co., Ltd.) was dissolved in 1,000 ml of tap water. The composition of the medium was peptone 1.5 %, meat extract 0.5 %, NaCl 0.5 % and K_2HPO_4 0.5 %, pH 7.0 before sterilization.

3. Growth of bacteria: The turbidity at 610 mμ was measured. The number of viable cells was determined by plating.

4. Leakage of cellular constituents: Growing cells were suspended in a fresh medium at appropriate dilutions and shaken in the presence or absence of the antibiotic at 30°C. At various time intervals, aliquots were withdrawn, cells were removed by centrifugation (3,000×g for 10 minutes), and the presence of 260 mμ absorbing material in the medium was determined.

5. Protein, DNA and RNA syntheses: Cells were grown overnight at 30°C, washed, resuspended with 20-fold dilution in a fresh medium and shaken. After a two-hour incubation, the drug was added to the medium. At appropriate time intervals, aliquots were withdrawn and the contents of RNA, DNA and protein were measured after SCHMIDT-THANNHAUSER-SCHNEIDER fractionations[3~5] by the absorbancies, at 260 mμ and 280 mμ for nucleic acids and protein, respectively.

6. Endogenous and exogenous respiration: Cells were grown overnight at 30°C,

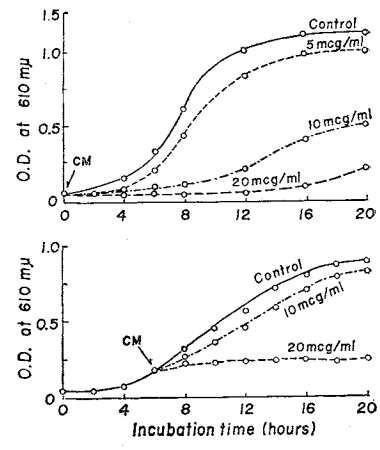
washed with saline, suspended in M/15 phosphate buffer, pH 7.0, and shaken for a few hours to reduce autorespiration; the cells were washed again and resuspended in the same buffer. A conventional WARBURG technique was used. A cell suspension (0.5 ml) of *ca.* 2 mg/ml dry weight was placed in the side arm. The main compartment contained 0.2 ml of 2.7 mg/ml glucose for exogenous respiration or buffer for endogenous respiration, 0.5 ml of the antibiotic, and 1.1 ml of the buffer. In the center well, 0.2 ml of 20 % potassium hydroxide was placed with a tip of filter paper. Gas phase was air. Incubation was carried out at 37°C, and the oxygen uptake was measured periodically.

7. Preparation of rat liver mitochondria: Rat liver mitochondria were prepared by the method of SCHNEIDER[6]. Rat liver was gently homogenized in a solution of 0.25 M sucrose, 10 mM potassium chloride, 0.2 mM EDTA, 5 mM magnesium chloride, 20 mM tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.2, and centrifuged at $700\times g$; the supernatant was centrifuged at $7{,}000\times g$ and the pellet was collected as the mitochondrial fraction. The oxidase activity was measured with an oxygen-meter[7] in a mixture of 2.75 ml of 0.25 M sucrose, 10 mM potassium chloride, 10 mM tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.2, 5 mM potassium phosphate buffer, pH 7.2, 5 mM magnesium chloride, 0.2 mM EDTA, 0.1 ml of mitochondrial suspension (*ca.* 5 mg protein), 0.05 ml of substrate (final 10 mM), 0.05 ml of ADP solution (final 0.6 mM), and 0.05 ml of inhibitor in a total of 3.0 ml at 25°C. The ATPase activity was measured as inorganic phosphorus released in the reaction medium containing 10 mM tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.2, 75 mM sucrose, 75 mM potassium chloride, 0.01 ml of water or cryomycin solution, and 0.05 ml of mitochondrial suspension (*ca.* 3 mg protein) in a total of 1 ml. The reaction was started by the addition of 5 μmoles of ATP. Incubation was carried out for 10 minutes at 30°C. Inorganic phosphorus was measured by the method of TAKAHASHI.[8]

8. Autolysis of *Bacillus* species: 0.5 ml of an overnight culture of each strain was added to 4.1 ml of fresh broth medium and incubated at 28°C. At the appropriate time of the exponential growth phase of the organism, 0.4 ml of cryomycin solution at a final concentration of 5 or 20 mcg/ml was added to the medium, and the incubation was continued. The turbidity was periodically measured at 610 mμ.

9. Autolysis of *Bacillus subtilis*: Preparation of autolytic enzymes: Two-day cultured broth filtrates of *B. subtilis* IFO 3037 were used as the source of the exocellular enzymes. A sonicate of the cells was used as the source of the endocellular enzymes. Growing cells of *B. subtilis* were suspended in M/15 potassium phosphate buffer, pH 7.0, containing 0.7 M sucrose; 0.5 ml of each autolytic enzyme solution or buffer as a control, and 0.5 ml of cryomycin solution at a final concentration of 0, 5 or 20 mcg/ml were added to 4 ml of the cell suspension. Incubation was carried out at 37°C. At appropriate time intervals, the turbidity at 610 mμ was measured.

Fig. 1. Effect of cryomycin on the growth of *Staphylococcus aureus* IFO 3061.



Results

1. Effect on Growth

Figs. 1 and 2 show that the growth of *Staphylococcus aureus* and *Bacillus subtilis* is inhibited by cryomycin. The minimum growth inhibitory concentrations against *S. aureus* and *B. subtilis* were 20 and 5 mcg/ml, respectively. Concentrations greater than 5 mcg/ml of cryomycin caused a rapid decrease of cell turbidity of *B. subtilis*.

Fig. 2. Effect of cryomycin on the growth of *Bacillus subtilis* IFO 3037.

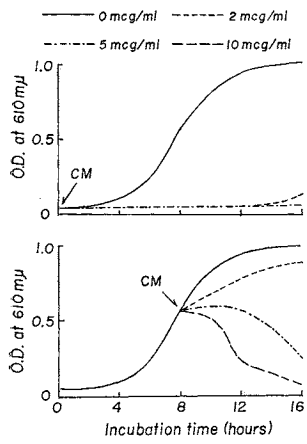
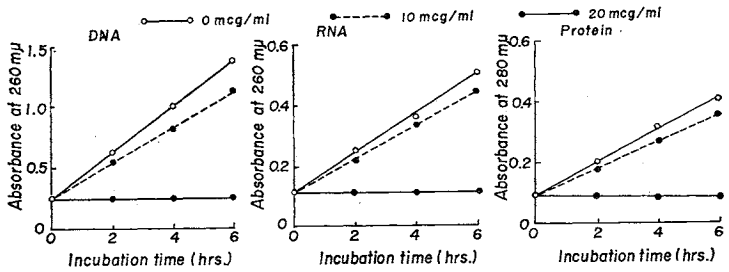


Table 1. Effect of cryomycin on cell growth and viability

Cryomycin (mcg/ml)	Length of incubation (hrs.)	Viable cells (per ml)	
		S. aureus	*B. subtilis*
0	0	4.8×10^8	1.1×10^8
0	1	5.5×10^8	5.9×10^8
0	3	1.1×10^9	1.3×10^9
10	1	6.2×10^8	1.9×10^6
10	3	5.0×10^8	4.8×10^7
20	1	4.2×10^7	4.8×10^6
20	3	3.7×10^7	9.6×10^5
40	1	4.8×10^6	4.8×10^6
40	3	1.6×10^5	8.0×10^5

As shown in Table 1, cryomycin showed a bactericidal action rather than a bacteriostatic one against the test organisms.

Fig. 3. Effect of cryomycin on nucleic acid and protein syntheses in *Staphylococcus aureus* IFO 3061.



2. Effect on the Syntheses of Protein and Nucleic Acids

Cryomycin inhibits the syntheses of protein and nucleic acids to almost the same degree in *S. aureus* (Fig. 3). The same results were obtained in *B. subtilis*.

3. Effect on Cell Membrane

Fig. 4 shows that cryomycin causes the leakage of cellular constituents from *B. subtilis*. Only slight leakage was observed in *S. aureus*.

4. Effect on Respiration

Cryomycin at its minimum growth inhibitory concentration affects the respiration of the sensitive test organisms. Oxygen uptake in cultures of *S. aureus* was stimulated immediately after the addition of cryomycin (Fig. 5). The same results were obtained in *B. subtilis*.

Inhibition of respiration by cryomycin was investigated using rat liver mitochondria and the oxygen-meter method. As shown in Fig. 6,

Fig. 4. Effect of cryomycin on leakage of cellular constituents.

Reaction system: 267 μmoles K.P.B. (pH 7.0), 2.8 mmoles sucrose, 4 mg cells in 4 ml; 30℃.

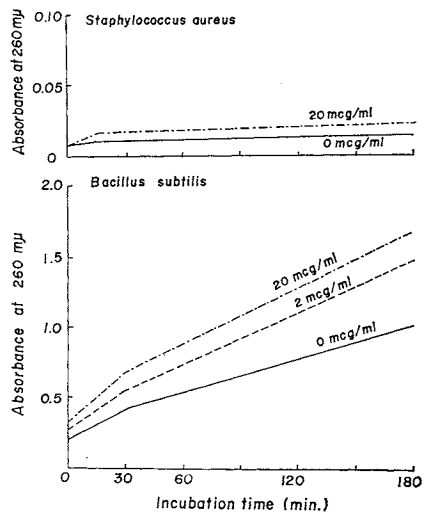


Fig. 5. Effect of cryomycin on respiration of *S. aureus*.

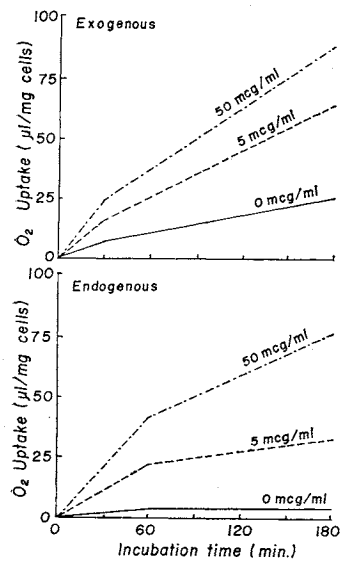


Fig. 6. Effect of cryomycin on the oxygen consumption of rat liver mitochondria.

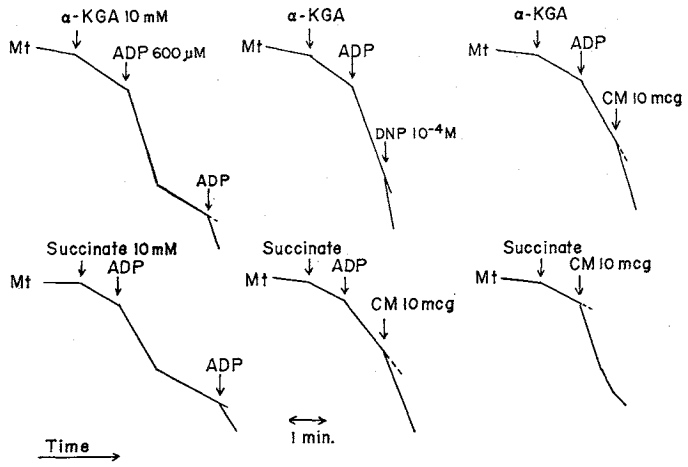


Table 2. Effect of cryomycin on ATPase

Cryomycin (mcg/ml)	Inorganic P released (μmoles)	
	Control*	$+10^{-5}$ M DNP
0	0.11	2.56
10	0.18	2.88

* The reaction systems are described in the text.

Table 3. Effect of cryomycin on the autolysis of *Bacillus* spp.

Microorganism	Autolysis	
	5 mcg/ml	20 mcg/ml
B. aneurinolyticus AKU 0201	±	++
B. natto AKU 0205	±	+
B. pumilus IFO 3028	±	+
B. roseus IAM 1257	±	++
B. subtilis IFO 3007	±	+
B. subtilis IFO 3026	−	++
B. subtilis IFO 3037	++	++
B. subtilis IFO 3009	±	++
B. subtilis IFO 3022	−	+
B. subtilis IAM 1193	±	++
B. subtilis var. *aterrimus* IFO 3214	−	++
B. subtilis var. *niger* IFO 3108	±	++
B. sphaericus IFO 3525	−	++
B. brevis IFO 3331	−	++
B. subtilis K wild Ajinomoto	+	++
B. licheniformis IAM 11054	±	+
B. subtilis Marburg W 23 AKU 0224	−	−
B. subtilis K AKU 0225	±	++
B. sphaericus IFO 3341	+	++
B. sphaericus IFO 3526	−	+
B. sphaericus IFO 3527	±	+
B. sphaericus IFO 3528	−	++

++ : Rapid autolysis (within 1 hour after addition of CM)
+ : Delayed autolysis (within 2 or 3 hours)
± : No autolysis (growth markedly inhibited)
− : No autolysis (growth gradually continued)

cryomycin stimulated respiration from 'step 3' to 'step 4' similarly to 2,4-dinitrophenol, a typical uncoupler of oxidative phosphorylation. Cryomycin did not activate ATPase (Table 2).

5. Effect on Autolysis of *Bacillus* Species

High concentrations of cryomycin were found to induce autolysis of most of the microorganisms belonging to the genus *Bacillus* (Table 3).

When endocellular or exocellular autolytic enzymes were added to a cell suspension of *B. subtilis*, a rapid decrease of turbidity was observed with and without the addition of cryomycin. Without the addition of the enzyme solution, the decrease of turbidity caused by the inhibitor was delayed (Fig. 7). Upon a microscopic observation, most of the cells had burst; no spore formation was observed.

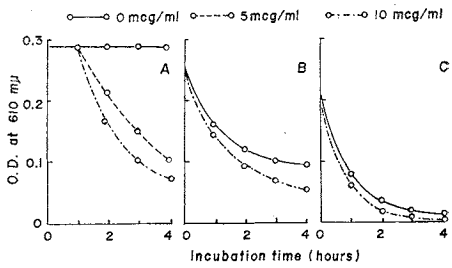
Discussion

We suggest that the primary action of cryomycin is on respiration, especially on the coupling between oxidative phosphorylation and electron transport. This conclusion is supported by the fact that the inhibitor does not promote the ATPase activity of mitochondria at its respiration-stimulating concentration.

Gramicidin[9] and valinomycin[10] are known to act as the uncouplers of oxidative phosphorylation, and their modes of action resemble those of cryomycin. The molecular weight of cryomycin is assumed to be *ca.* 4,000 (calculated as minimal molecular weight); the concentration causing the uncoupling is 10^{-6}~10^{-5} M.

Fig. 7. Effect of cryomycin on autolysis of *Bacillus subtilis* IFO 3037.

A : Enzymes free B : Endocellular enzymes added. C : Exocellular enzymes added. Temperature : 37℃



Since the respiration site of bacteria is believed to be located near the cell membrane, we would like to suggest that the abnormal respiration stimulated by cryomycin causes disruption of the cell barrier followed by lysis of the *Bacillus* species tested in these experiments.

Further studies on the mechanism of action of cryomycin on respiration and the correlation between the stimulated respiration and cell autolysis of *B. subtilis* will be presented in a subsequent publication.

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