THE MODE OF ACTION OF CERVINOMYCIN IN *STAPHYLOCOCCUS AUREUS*

HARUO TANAKA, KAZUHITO KAWAKITA, HIDEKAZU SUZUKI, PRISKA SPIRI-NAKAGAWA and SATOSHI ŌMURA*

School of Pharmaceutical Sciences, Kitasato University, and The Kitasato Institute, Minato-ku, Tokyo 108, Japan

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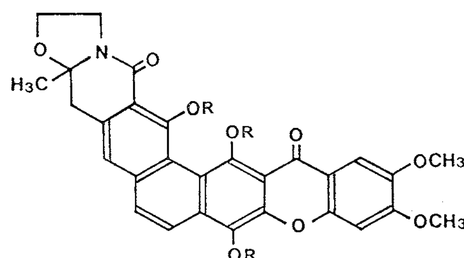
The mode of action of cervinomycin, which is a new antibiotic active against Gram-positive bacteria including anaerobes, was studied in *Staphylococcus aureus* using triacetylcervinomycin A_1 (ACVM), an acetyl derivative of cervinomycin A_1. ACVM inhibited strongly the growth of the organism when it was added to a culture at the time of inoculation at a concentration of 1.0 μg/ml, but did not inhibit when added to a logarithmic phase culture even at 10.0 μg/ml. The antibiotic also inhibited the incorporation of labeled precursors of cell wall peptidoglycan (*N*-acetylglucosamine), RNA (uridine), DNA (thymidine) and protein (L-leucine) into both whole cell and acid-insoluble macromolecular fractions. ACVM stimulated the leakage of UV_{260}-absorbing materials, amino acids and potassium ions from resting cells and protoplasts. Phospholipids partially reversed the inhibitory activity of ACVM in a growing culture. These findings suggest that ACVM interact with phospholipids in the cytoplasmic membrane and then interfere with the membrane transport system.

Cervinomycins A_1 and A_2 are new antibiotics containing a xanthone structure which are produced by *Streptomyces cervinus* Takahashi and Ōmura sp. nov. AM-5344.[1~3] They are highly active against Gram-positive bacteria including *Staphylococcus*, *Bacillus*, some anaerobes (*Clostridium*, *Peptococcus*, *Bacteroides etc.*) and mycoplasmas.[1,4] Triacetylcervinomycin A_1 (ACVM, Fig. 1) among acetyl derivatives possesses higher solubility and low toxicity in addition to a more potent antimicrobial activity.

The present paper deals with the mode of action of ACVM in *Staphylococcus aureus*.

Fig. 1. The structures of cervinomycin A_1 and triacetylcervinomycin A_1 (ACVM).



Cervinomycin A_1 R=H
ACVM R=$COCH_3$

Materials and Methods

Bacterial Strain

Staphylococcus aureus FDA 209P (KB 34) maintained in this laboratory was used.

Growth of the Organism

A loopful of cells of the bacterium, which had been kept on Brain Heart Infusion Agar (Difco), was transferred into a Monod tube containing 10 ml of Nutrient Broth (Difco, pH 7.0) and incubated on a Monod shaker for 16~20 hours at 37°C to give a seed culture. An appropriate volume of the culture was transferred into a Monod tube containing 10 ml of Brain Heart Infusion Broth (Difco, pH 7.4) to make an OD_{660} of 0.05, and incubated at 37°C with shaking.

To investigate the effect of ACVM on the growth of the organism, various concentrations of ACVM dissolved in MeOH were added aseptically to a culture at zero time or to a logarithmic phase

culture. Growth was monitored by OD_{660} which was measured with a Coleman Junior II spectrometer.

Incorporation of Biosynthetic Precursors of Cellular Macromolecules into Whole Cell or Acid-insoluble Fractions

L-[*U*-^{14}C]Leucine, [*methyl*-^{3}H]thymine, [2-^{14}C]uridine and *N*-acetyl-D-[1-^{3}H]glucosamine were used as precursors for synthesis of protein, DNA, RNA and cell wall peptidoglycan, respectively. After *S. aureus* was cultured in a Monod tube at 37°C to reach the logarithmic phase (OD_{660}=0.1), radioactive precursors (aqueous solution, 10 μl) at a final concentration of 0.5 μCi/ml and ACVM (MeOH solution, 50 μl) at various concentrations were added to 5.0 ml of the logarithmic phase culture, and incubated at 37°C with shaking.

For the determination of incorporation of the precursors into *S. aureus* cells, an aliquot (0.5 ml) of the culture was filtered through a membrane filter (Toyo TM-2, pore size 0.45 μm) after incubation for 1, 3, 5 and 10 minutes. The cells collected on the filter was washed 3 times with 10 ml of 0.05 M bis-Tris-HCl buffer (pH 7.0). The radioactivity of the cells on the filter was counted with an Aloka liquid scintillation spectrometer using ACS II (Amersham). For the determination of incorporation of the precursors into acid-insoluble macromolecular fraction in the cells, an aliquot (0.5 ml) of the culture was added to 10 ml of ice-cold 5% TCA. The resultant precipitate was collected on a membrane filter and washed 3 times with 10 ml of TCA. The radioactivity of acid-insoluble fraction on the filter was counted with a liquid scintillation spectrometer.

Release of Contents from *S. aureus* Cells

The cells in a logarithmic phase culture (OD_{660}=0.1) were harvested by centrifugation at 2°C for 10 minutes at 1,700×*g*, washed with 0.05 M bis-Tris buffer (pH 7.0) and then suspended in the same buffer to give OD_{660}=0.05. To 100 ml of the suspension ACVM was added at a final concentration of 0.1, 1.0 or 10.0 μg/ml. The mixture was incubated at 37°C with shaking. An aliquot (10 ml) of the mixture during incubation was taken out and subjected to determinations of OD_{660}. The filtrate passing through a membrane filter (Toyo TM-2, pore size 0.45 μm) was washed 3 times with an equal volume of ethyl acetate and then submitted to determinations of UV-absorption at 260 nm, protein, amino acids, sugars and K^+ and Na^+ ions. The concentrations of protein, amino acids and sugars were determined by the colorimetric methods as described by SMITH *et al.*,[5] MOORE and STEIN,[6] and HODGE and HOFREITER,[7] respectively. The K^+ and Na^+ concentrations were estimated by the atomic absorption method with a Shimadzu atomic absorption flame emission spectrophotometer AA 640-12.

Preparation of Protoplasts

The cells of *S. aureus* grown in Brain Heart Infusion Broth at 37°C were harvested by centrifugation in the logarithmic phase (OD_{660}=0.2), washed twice with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M NaCl and 0.5 M sucrose and suspended in the same buffer to give a cell suspension of OD_{660}=0.5. To the cell suspension, 250 U of achromopeptidase from *Achromobacter lyticus* was added and incubated at 37°C for 1~2 hours with shaking. When the OD_{660} of the cell suspension decreased by half (to 0.25), most of the cells had been converted into protoplasts. Formation of protoplasts was observed under a phase-contrast microscope. After the remaining intact cells were removed by centrifugation at 2°C for 10 minutes at 100×*g*, the protoplasts were harvested by centrifugation at 2°C for 15 minutes at 2,600×*g*, and suspended in 75 ml of the above buffer containing 0.01 M NaCl and 0.5 M sucrose.

Release of UV-absorbing Materials from Protoplasts

To the protoplast suspension (35 ml), ACVM was added at a final concentration of 20 μg/ml and incubated at 37°C with shaking. At appropriate time intervals, the OD_{660} of the suspension was measured and the supernatant obtained by centrifugation at 2,600×*g* for 15 minutes was washed twice with ethyl acetate and subjected to determination of A_{260}.

Reversal of Antimicrobial Activity of ACVM

Reversal by phospholipids, fatty acids, sugars, bovine serum albumin and horse serum of the

anti-*Staphylococcus* activity of ACVM was examined by the conventional paper disc method using Nutrient Agar (Difco) as test medium and *S. aureus* as test organism. In the case of a growing culture, these additives were added after the culture was preincubated with 1.0 μg/ml of ACVM at 37°C for 30 minutes.

Aggregation of Phospholipids Vesicles

Four kinds of phospholipid vesicles were prepared by the method of YUNG and GREEN[8] with minor modification. To prevent a too rapid aggregation of negatively charged vesicles, a mixture of phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) or cardiolipin (CL) with phosphatidylcholine (PC) (1 : 4, w/w) was used rather than a single compound. The phospholipid mixture prepared in 0.05 M bis-Tris buffer (pH 7.0) was treated with sonication (100 W, 1 minute, 5 times) to disperse unilamellar phospholipid vesicles. The mixture was centrifuged at 100,000 × *g* for 30 minutes and the supernatant fluid was used for the aggregation assay. Aggregation of vesicles was determined by the change in OD_{660} during 2 minutes after addition of ACVM.

Antibiotic and Chemicals

ACVM was prepared from cervinomycin A_1 as described by ŌMURA *et al.*[2] L-[*U*-^{14}C]Leucine (342 mCi/mmol) was purchased from Amersham. [*Methyl*-^{3}H]thymidine (2.85 Ci/mmol), [2-^{14}C]-uridine (50 mCi/mmol) and *N*-acetyl-D-[1-^{3}H]-glucosamine (3.42 Ci/mmol) were purchased from New England Nuclear. Phospholipids were purchased from Sigma. Other chemicals were also obtained commercially.

Results

Effect of ACVM on Growth

The effect of ACVM on the growth of *S. aureus* FDA 209P was examined. When ACVM (0.1 μg/ml) was added before growth (OD_{660}= 0.05), it significantly suppressed the increase of OD_{660}, and 1.0 μg/ml of ACVM completely inhibited growth (Fig. 2A). These data are in agreement with the MIC (0.2 μg/ml) of ACVM against the organism on Heart Infusion Agar. On the other hand, when ACVM was added to an exponentially growing culture (OD_{660}=0.1),

Fig. 2. Effect of ACVM on growth of *Staphylococcus aureus*.

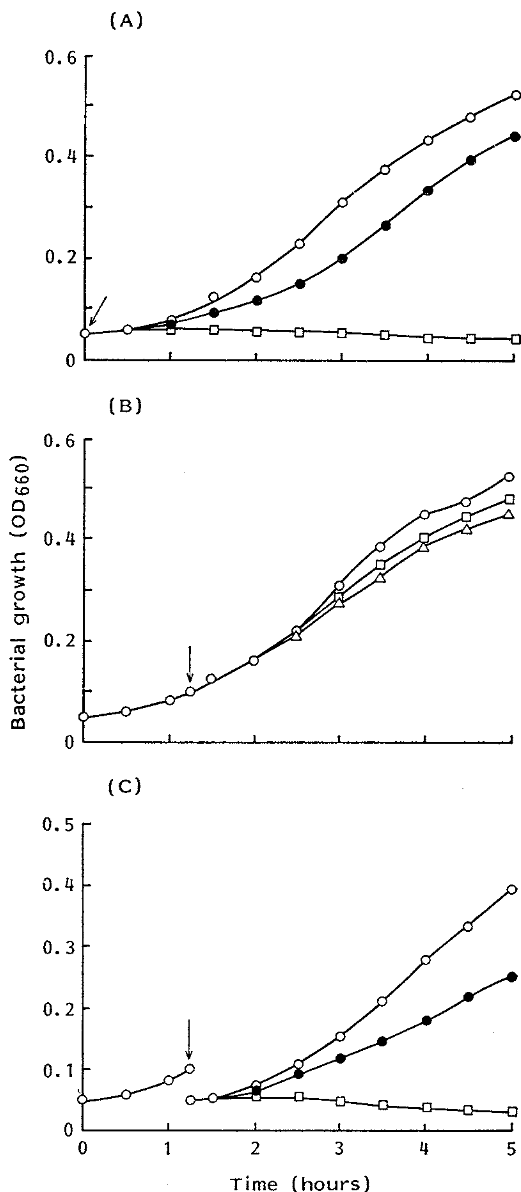
○ None, ● ACVM 0.1 μg/ml, □ ACVM 1.0 μg/ml, △ ACVM 10.0 μg/ml.

Each arrow indicates addition of ACVM.

(A) ACVM was added at start of incubation (OD_{660}=0.05).

(B) ACVM was added during the exponential growth phase.

(C) ACVM was added after dilution of a growing culture from the exponential growth phase to OD_{660}=0.05.



ACVM at a final concentration of 10.0 μg/ml hardly affected growth (Fig. 2B). When ACVM was added to an exponentially growing culture which had been diluted to OD_{660}=0.05 corresponding to that at zero time, however, it inhibited growth at a final concentration of 1.0 μg/ml (Fig. 2C). The above results indicate that the inhibition depends on the absolute concentration of cells, and not simply on the ratio between antibiotic and cells.

Effect of ACVM on Incorporation of Biosynthetic Precursors of Cellular Macromolecules into Whole Cell and Acid-insoluble Fractions

The effect of ACVM on the incorporation of precursors for macromolecular synthesis, [*methy*-^{3}H]thymidine, [2-^{14}C]uridine, L-[*U*-^{14}C]leucine and *N*-acetyl-D-[1-^{3}H]glucosamine, both into whole cells and into acid-insoluble fractions of *S. aureus* was studied. As shown in Fig. 3, ACVM inhibited the incorporation of all of the precursors into the cells. The incorporation of [^{3}H]GlcNAc and [^{14}C]-uridine for 10 minutes was almost completely inhibited at 0.1 μg/ml, and that of [^{14}C]leucine and [^{3}H]-thymidine was inhibited by 80 and 60%, respectively, at 1.0 μg/ml. The incorporation of these precursors into the acid-insoluble fraction was inhibited by ACVM in parallel with that into the cells as shown in Fig. 4.

When the degree of inhibitory effect on incorporation of precursors into the cells was compared

Fig. 3. Effect of ACVM on the uptake of biosynthetic precursors of macromolecules into *Staphylococcus aureus* cells.

(A) [^{14}C]Uridine, (B) [^{3}H]GlcNAc, (C) [^{3}H]thymidine, (D) [^{14}C]leucine.
○ None, ● ACVM 0.1 μg/ml, □ ACVM 1.0 μg/ml.

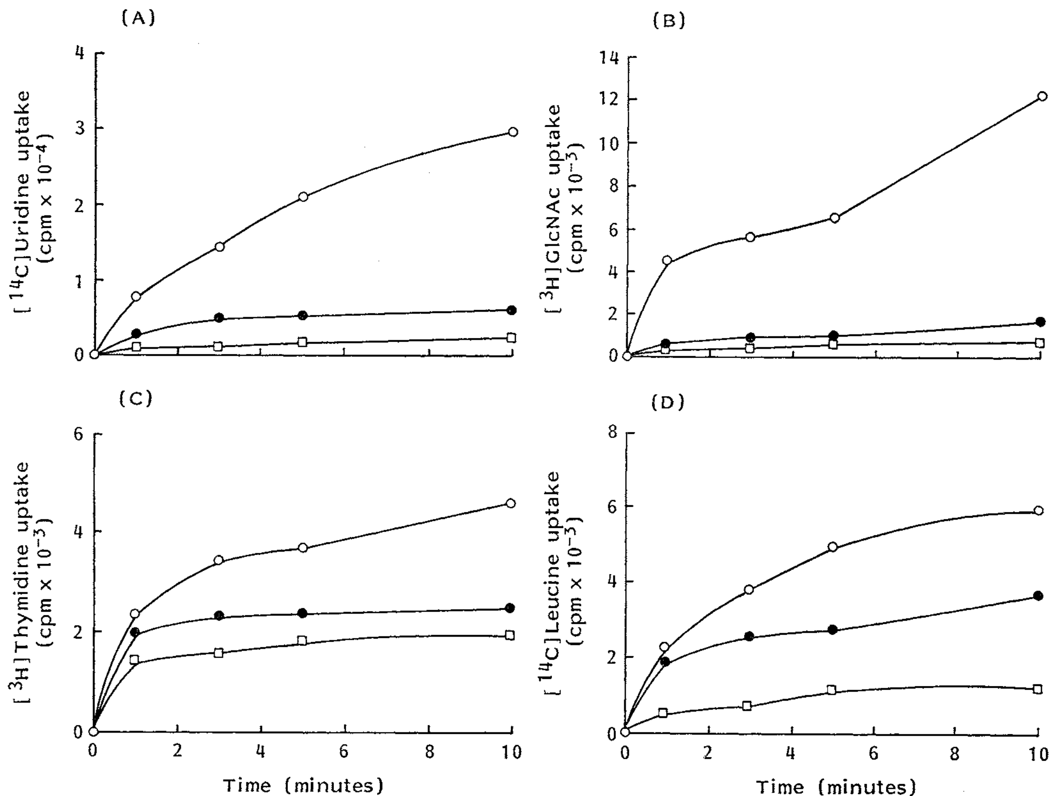
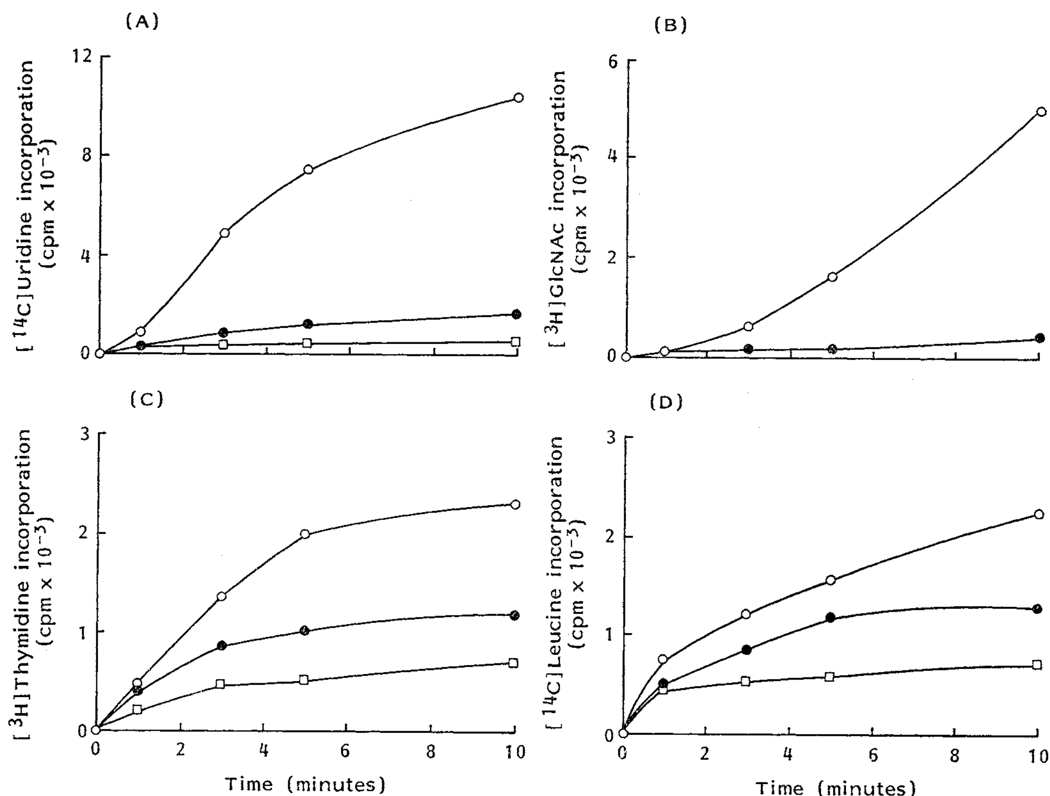


Fig. 4. Effect of ACVM on the incorporation of precursors into the acid-insoluble macromolecular fraction in *Staphylococcus aureus*.

(A) [^{14}C]Uridine, (B) [^{3}H]GlcNAc, (C) [^{3}H]thymidine, (D) [^{14}C]leucine.
○ None, ● ACVM 0.1 μg/ml, □ ACVM 1.0 μg/ml.



with that into the acid-insoluble fraction at the same concentration of ACVM, the former was found to be equivalent to or higher than the latter. When the comparisons were made in a short time interval (*e.g.* 1 minute), the former was much higher than the latter, especially in the cases of [^{3}H]GlcNAc (94.3% inhibition for cells, 37.2% inhibition for acid-insoluble fraction), [^{14}C]uridine (85.5%, 61.8%, respectively) and [^{14}C]leucine (78.2%, 37.9%, respectively).

These results suggest that the inhibition of uptake of the precursors into the cells results in the decrease of incorporation into the acid-insoluble macromolecular fraction.

Effect of ACVM on Release of Various Constituents from *S. aureus* Cells

It is known that some membrane-active antibacterial agents produce a rapid loss of materials from the metabolic pool of the cell[9~12]. Thus, we examined the effect of ACVM on release of metabolites from *S. aureus* cells. When the cell suspension in 0.05 M bis-Tris buffer (pH 7.0) was incubated in the presence of ACVM (1.0 μg/ml), the antibiotic stimulated the release of UV_{260}-absorbing materials, amino acids and K^+ ions from the cells (Fig. 5), but did not stimulate that of proteins, sugars and Na^+ ions even at a concentration of 10.0 μg/ml. During incubation, a decrease in OD_{660} of the cell suspension was not observed (the data not shown), indicating that the release of UV-absorbing materials, amino acids and K^+ ions is due not to lysis but to leakage from cells.

Fig. 5. Effect of ACVM on release of UV_{260}-absorbing materials (A), amino acids (B) and potassium ions (C).

○ None, ● ACVM 0.1 μg/ml, □ ACVM 1.0 μg/ml, △ ACVM 10.0 μg/ml.

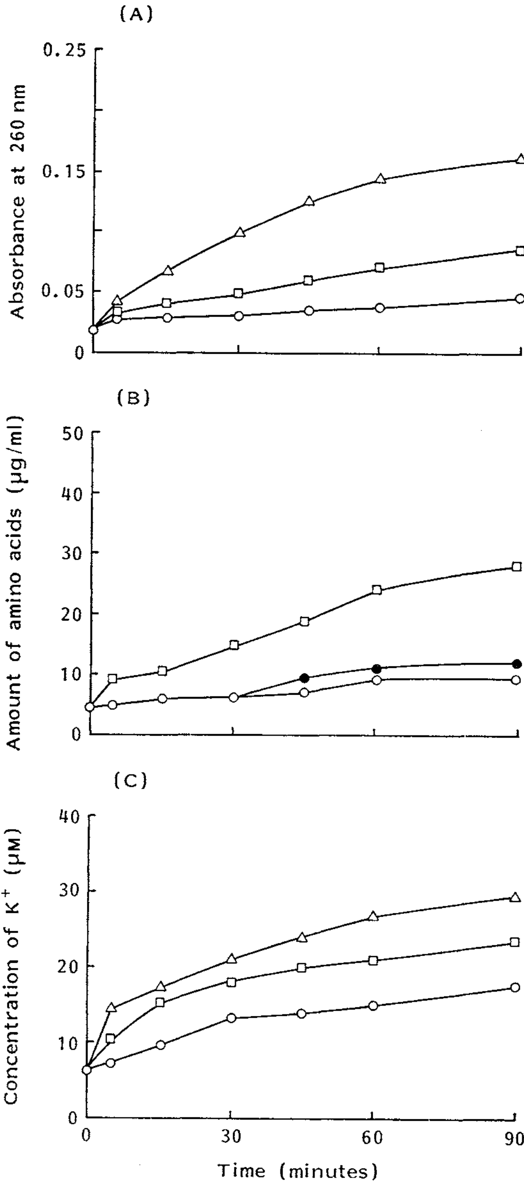


Fig. 6. Effect of ACVM on release of UV_{260}-absorbing materials from protoplasts of *Staphylococcus aureus*.

○ None, □ ACVM 2.0 μg/ml, △ ACVM 20.0 μg/ml.

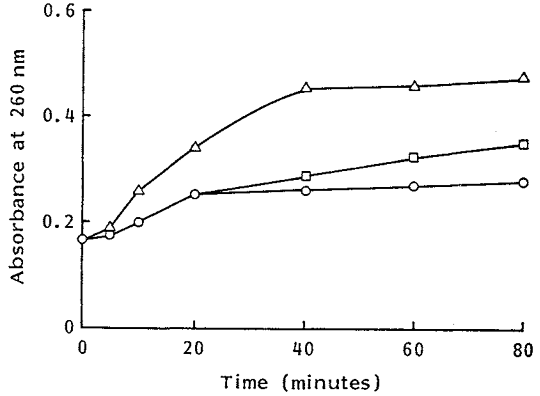
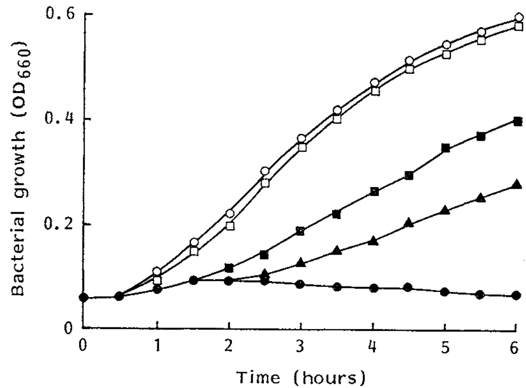


Fig. 7. Reversal by phospholipids (PL) of the growth inhibition of *Staphylococcus aureus* by ACVM.

○ None, ● ACVM 1.0 μg/ml, ▲ PL 0.3 mg/ml+ACVM 1.0 μg/ml, ■ PL 0.5 mg/ml+ACVM 1.0 μg/ml, □ PL 0.5 mg/ml.



Effect of ACVM on Release of UV-absorbing Materials from Protoplasts

When a protoplast suspension of *S. aureus* in 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M NaCl and 0.5 M sucrose was incubated at 37°C, the release of UV_{260}-absorbing materials was stimulated by the addition of ACVM (2.0 μg/ml) as shown in Fig. 6. During incubation for 80 minutes, lysis of protoplasts was not observed.

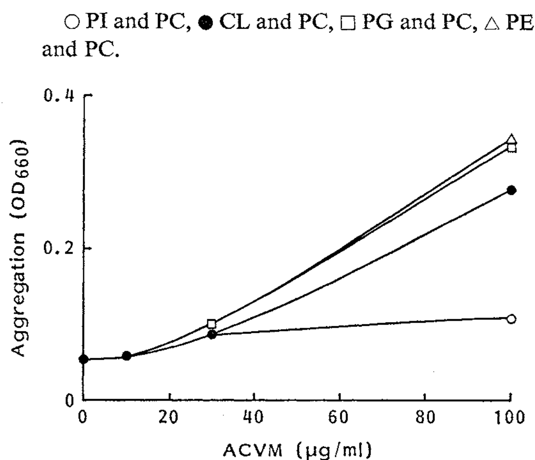
Reversal of Antimicrobial Activity of ACVM by Phospholipids and Horse Serum

Among various substances tested (phospholipids, fatty acids, sugars, bovine serum albumine and horse serum), phospholipids and horse serum showed a reversal effect of the antimicrobial activity of

ACVM against *S. aureus* in the conventional paper disc method (data not shown). When a mixture of PG, PI, CL, PE and PC (1 : 1 : 1 : 1 : 4, w/w) was added to a growing culture of the organism at 0.3 or 0.5 mg/ml, the growth which was inhibited by ACVM (1.0 μg/ml) was partially restored by about 40 or 60%, respectively, as shown in Fig. 7. These results suggest the interaction between ACVM and phospholipids in cytoplasmic membrane.

Fig. 8. Aggregation of phospholipid vesicles in the presence of ACVM.

○ PI and PC, ● CL and PC, □ PG and PC, △ PE and PC.



Aggregation of Phospholipid Vesicles in the Presence of ACVM

ACVM at a high concentration was found to cause aggregation of the phospholipid vesicles composed of PG/PC, PE/PC or CL/PC (1 : 4, w/w). As shown in Fig. 8, the vesicles containing PE or PG were aggregated more easily than those containing CL when they were incubated with 100 μg/ml of ACVM for 2 minutes. In contrast, vesicles containing PI hardly aggregated.

Discussion

ACVM at 1.0 μg/ml inhibited completely the growth of *S. aureus* when added at zero time, but it hardly inhibited when added during the exponential phase; the inhibition depended on the absolute concentration of cells in the culture, and not on the ratio of antibiotic to cells. These results indicate that ACVM acts strongly the lag phase cells.

ACVM inhibited the incorporation of the precursors of cellular macromolecules, *i.e.* GlcNAc, uridine, thymidine and leucine, both into whole cells and acid-insoluble fractions in the organism. These findings suggest two possibilities. One is that ACVM acts on the cell membrane and interferes with transport systems. The other is that ACVM acts on energy metabolism. The inhibitory rates of incorporation of precursors into cells were higher than those into acid-insoluble macromolecular fraction especially in the incorporation of [^{3}H]GlcNAc, [^{14}C]uridine and [^{14}C]leucine in a short time (*e.g.* 1 minute) (Figs. 3 and 4). This seems to indicate that ACVM interferes with the active transport system in cytoplasmic membrane predominantly, although the effect on energy metabolism is not eliminated.

Furthermore, ACVM caused the leakage of UV-absorbing materials, amino acids and K^+ ions from the cells (Fig. 5) and also of UV-absorbing materials from protoplasts (Fig. 6). For the leakage of such metabolites, a higher concentration of ACVM was necessary than for the inhibition of the incorporation of biosynthetic precursors. We also found that phospholipids are able to reverse partially the inhibition of the growth (Fig. 7) and the aggregation of phospholipid vesicles is caused by ACVM *in vitro* though the latter needs a relatively high concentration of ACVM (Fig. 8).

These findings suggest that ACVM interacts with phospholipids, as reported for membrane active agents such as polymyxin[13] and surfactin.[14] However, further investigation is necessary to determine whether or not ACVM binds to the phospholipids of the cytoplasmic membrane.

Consequently, it is considered that the antibiotic primarily acts on the cytoplasmic membrane and interferes with active transport systems at a low concentration (*e.g.* 0.1 μg/ml). This results in the inhibition of incorporation of biosynthetic precursors and growth. Release of cellular metabolites such as amino acids, K^+ ions and UV-absorbing materials is caused at a higher concentration (*e.g.* over 1.0 μg/ml).

Several polycyclic xanthone antibiotics, *e.g.* albofungin,[15] lysolipin I[16] and actinoplanones[17,18] are known, but the above-mentioned mode of action of ACVM seems to be different from these other antibiotics. Albofungin, active against Gram-positive bacteria and fungi but much less active againt Gram-negative bacteria, inhibits RNA synthesis and to a lesser extent, protein synthesis in *Candida albicans*, suggesting that the antibiotic action is based on its inhibition of DNA-dependent RNA synthesis.[19] Lysolipin I, active against Gram-positive and Gram-negative bacteria and weakly active against fungi, acts lytically against bacterial cells and the activity is decreased by several lipids. Its site of action was speculated to be the biosynthesis of bacterial cell walls, an interaction with the carrier lipid for peptidoglycan intermediates being probable.[20] Recently, actinoplanone A was reported to possess a strong degree of cytotoxicity against HeLa cells with inhibitory action against DNA synthesis.[18] Direct comparison of the mode of action among them will however be necessary for further comparison of their modes of action.

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