

ON THE MODE OF ACTION OF HODYDAMYCIN

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The present study was conducted to determine how hodydamycin inhibits microbial growth. Hodydamycin had no effect on either the cell membrane or the synthesis of the cell wall of susceptible organisms. The antibiotic inhibited protein synthesis through its scissating effect on DNA. No effect on the synthesis of RNA was observed.

The peptide antibiotic hodydamycin was first isolated by SHIMI *et al.*¹⁾ from the fermented broth of *Streptomyces* AS-Y-400. The antibiotic possesses substantial activities against gram-positive bacteria, while its effect on most gram-negative organisms is minimal.

The present paper describes studies conducted on the mode of action of hodydamycin.

Materials and Methods

Organisms and culture conditions: *Bacillus subtilis* icc, *Staphylococcus aureus* (Oyama), and *Klebsiella pneumoniae* were grown in 200 ml nutrient broth having the following composition (g/100 ml) meat extract 0.15, yeast extract 0.15, peptone 0.5 and NaCl 0.5 in shaking cultures (220 rpm) at 35°C and harvested during the logarithmic phase at half maximal growth (optical density 1.0 at 660 m μ). The cells were collected by centrifugation at 15,000 *g* for 10 minutes and washed twice with sterile saline solution and were used for the following experiments.

Release of cellular material absorbing at 260 m μ : The method applied was that described by MIKIKO and YASUO²⁾. Intracellular substances from *S. aureus* were subjected to ultracentrifugation at 147,200 *g* using a MOM ultracentrifuge model 3170 for comparing the sedimentation pattern with non-treated cultures.

Preparation of protoplasts and spheroplasts: Washed cells of *B. subtilis* and *S. aureus* were suspended so as to attain a concentration of 0.08~0.1 mg dry weight of cells/ml in phosphate buffer (pH 7.2) containing 0.5 M sucrose and 100 μ g/ml of lysozyme. The suspensions were separately incubated for 90 minutes at 30°C. The formation of protoplasts was confirmed microscopically. All preparations were then used immediately. Spheroplasts of *K. pneumoniae* were prepared according to the method of REPASKE.³⁾

Determination of the lysing properties on protoplasts and spheroplasts: The effect of hodydamycin was determined as follows: 2.7 ml of a suspension of protoplasts (or spheroplasts in the case of *K. pneumoniae*) (ca. 0.08 mg dry weight/ml of the final solution, 2×10^8 cell/ml), and 0.3 ml of a solution containing hodydamycin were added to 0.5 M sucrose solution to give a final volume of 6 ml. The mixture was incubated at 37°C and the absorbance at 660 m μ was measured at 10-minute intervals for 40 minutes.

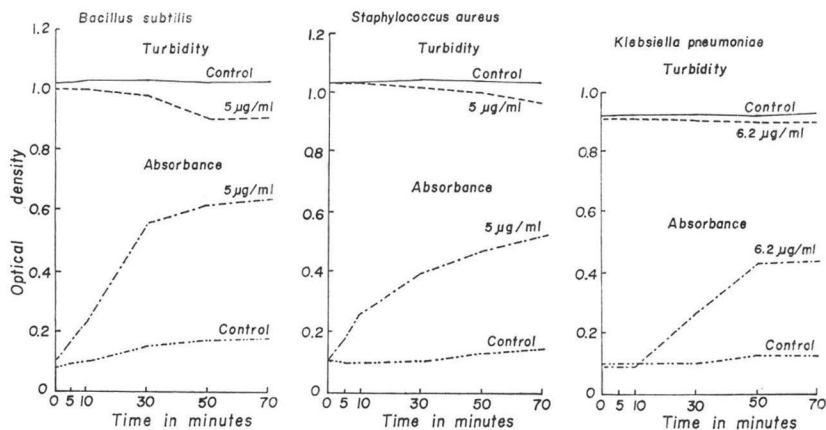
Determination of N-acetyl aminosugars in the cell wall of *S. aureus* was carried out by the method of STROMINGER.⁴⁾ Total protein was assessed by the FOLIN reagent.⁵⁾ The fractionation of DNA and RNA was carried out as described by SCHMIDT-THANNHAUSER.⁶⁾ RNA content of cells was determined by the orcinol method⁷⁾ and that of DNA by the indole method.⁸⁾

Degradation of DNA in intact cells of *S. aureus*: Washed cells were incubated with C¹⁴-thymine (1.0 uci/ml) containing medium for 2 hours, collected by centrifugation at 15,000 *g*,

washed with 300 ml of tris minimum medium⁹⁾ and finally resuspended in the growth medium containing unlabeled thymine (10 $\mu\text{g}/\text{ml}$). One ml portions of the cell suspension (*ca.* 1×10^8 cells) (3,840 cpm in cold TCA-insoluble fraction) were supplemented with various concentrations of hodydamycin and then incubated at 37°C. Samples of 0.1 ml were withdrawn at different intervals and treated with 2 ml of 5% cold TCA. The precipitates were washed twice with the cold TCA solution. The washings and supernatant after the TCA precipitation were admixed and then extracted with diethyl ether to remove the TCA. Radioactivity was assessed by a liquid scintillation spectrometer with a conventional toluene scintillation mixture.

Fig. 1. Effect of hodydamycin on growth and release of materials absorbing at 260 $m\mu$ of test organisms

Microbial cells harvested at the logarithmic phase, washed with saline solution and then suspended in phosphate buffer of pH 7.2 (*ca.* 1×10^8 cell/ml). Hodydamycin was supplemented to the cell suspensions and then latter incubated at 30°C for 70 minutes. Samples were withdrawn at intervals and the turbidity was assessed at 660 $m\mu$ while the absorbance measurements at 260 $m\mu$ was conducted for the supernatant after centrifuging the samples at 15,000 *g*.



Results

Incubating the cells of *B. subtilis*, *S. aureus* and *K. pneumoniae* with hodydamycin at the MIC level (5.0, 5.0 and 6.2 $\mu\text{g}/\text{ml}$ respectively) caused the release of 260 $m\mu$ -absorbing material (Fig. 1). The UV spectrum of these released materials (Fig. 2) is in accord with that of nucleic acids. The ultracentrifugal patterns of the substances released from *S. aureus* in presence and absence of the antibiotic are shown in Plates 1 a, b, c and d. The appearance of more than one peak (indicating compounds of different molecular weights) in treated cultures at this high field of gravity is probably due to unequal scission induced by hodydamycin on the DNA strands of the test organism.

Fig. 2. Absorption of released intracellular substances induced by hodydamycin
A *B. subtilis*, B *S. aureus*, C *K. pneumoniae*

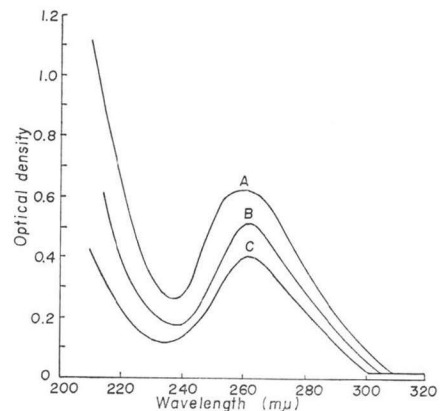


Plate 1. Sedimentation profile of the released materials from *S. aureus* cells

a and b: Sedimentation profile (SP) of control cultures (no hodydamycin) at 0.0 time (a) and after 20 minutes (b).

c and d: S.P. of cultures treated with the antibiotic (5 $\mu\text{g}/\text{ml}$) at 0.0 time (c) and after 20 minutes (d).

Photographs taken with Schlieren optics at 45,000 rpm with the phase plate at angle 60° and 20°C

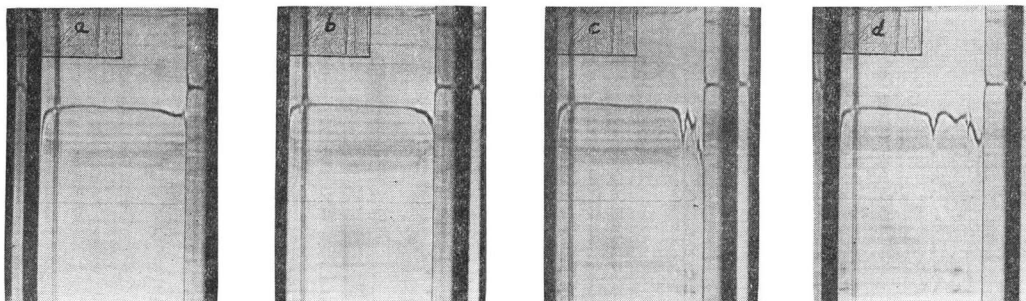
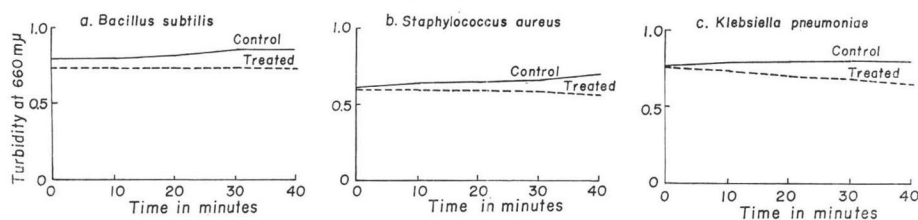


Fig. 3. Effect of hodydamycin on the protoplasts and spheroplasts of the test organisms



The results shown in Figs. 3 a, b and c demonstrate that the antibiotic at concentrations of 100 and 200 $\mu\text{g}/\text{ml}$ failed to lyse sphaeroplasts and protoplasts respectively suspended in hypertonic solutions. This failure to lyse is presumably due to the antagonistic effect of the hypertonic solution.* Hodydamycin when added to cultures of *S. aureus* at the minimum inhibitory concentration of 5 $\mu\text{g}/\text{ml}$ and at the higher concentration of 100 $\mu\text{g}/\text{ml}$ did not induce accumulation of N-acetyl aminosugars (Fig. 4).

The data presented in Fig. 5 c indicate that hodydamycin inhibited protein synthesis in growing cultures of *S. aureus* when the antibiotic was added at a concentration of 5 $\mu\text{g}/\text{ml}$. Increasing the concentration of hodydamycin to 10 $\mu\text{g}/\text{ml}$ resulted in a more marked effect. On the other hand the lack of an effect of the antibiotic on the RNA content was noted (Fig. 5 a). Finally a marked decrease

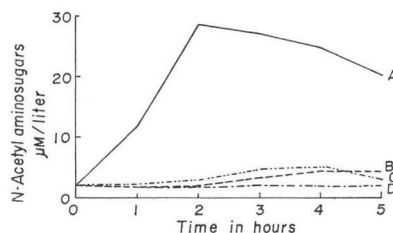
Fig. 4. Effects of hodydamycin and penicillin on the accumulation of N-acetylglucosamine in cultures of *S. aureus*.

A Culture supplemented with 5 $\mu\text{g}/\text{ml}$ of Na benzyl penicillin.

B Culture supplemented with 5 $\mu\text{g}/\text{ml}$ of hodydamycin.

C Culture supplemented with 100 $\mu\text{g}/\text{ml}$ of hodydamycin.

D Control culture (no antibiotic).



* GUZE, L. B.: Microbial Protoplasts, Sphaeroplasts and L-Forms. Williams & Wilkins Co., Baltimore, 1968. page 309, lines 8~14.

in the cellular DNA was observed (Fig. 5 b). In Table 1 the data show a notable elevation in the counts of cold 5% TCA-soluble fraction. This effect was markedly augmented by increasing concentrations of hodydamycin.

Fig. 5. Effect of hodydamycin on formation of DNA, RNA and protein in *Staphylococcus aureus*

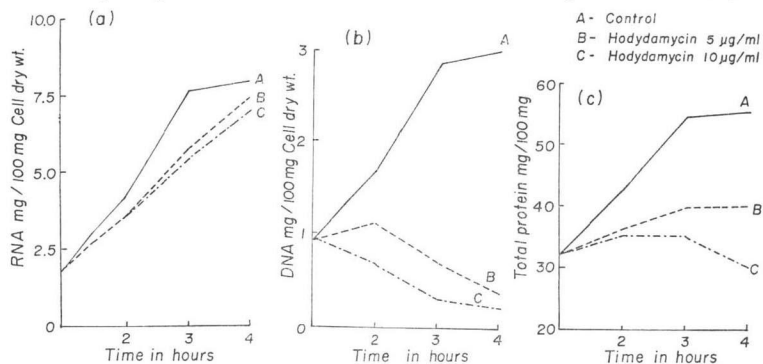


Table 1. Effect of hodydamycin on DNA breakdown in *S. aureus*

Concentration of hodydamycin ($\mu\text{g/ml}$)		Radioactivity (cpm/ml)			
		5 min.	20 min.	40 min.	60 min.
0	S	0	10	34	58
	R	3,830	3,829	3,783	3,755
1	S	0	20	503	1,010
	R	3,832	3,802	3,302	2,800
5	S	6	82	811	1,832
	R	3,825	3,755	3,025	2,000
7.5	S	5	127	800	2,154
	R	3,827	3,713	3,010	1,676
10	S	9	142	901	2,640
	R	3,823	3,696	2,905	1,204
15	S	20	152	1,207	2,920
	R	3,782	3,675	2,610	912

S: cpm of 5% TCA-soluble fraction of harvested cells of *S. aureus* present in 1 ml of culture.

R: cpm of 5% TCA-insoluble fraction of the same cells (residual).

Discussion

Hodydamycin had no effect on the cell membrane of sensitive organisms nor did it cause accumulation of N-acetyl glucosamine in *S. aureus* cells.

The antibiotic inhibited the synthesis of protein in *S. aureus* and caused a marked decrease in the DNA content. This effect was markedly augmented by increasing concentrations of hodydamycin.

Ultracentrifugation of intracellular material from treated *S. aureus* cells revealed the presence of two main fractions S-30 and S-50 both lacking in the control cultures. This may be due to asymmetric scission caused by the antibiotic on the DNA strands of *S. aureus*. This coupled with the observation that radioactivity present in the cold TCA-insoluble fraction was markedly

reduced by treatment with hodydamycin confirms that the antibiotic caused scission of the DNA. These fragments so produced could exude from inside the cell resulting in a detectable increase in radioactivity in the supernatants.

One can therefore conclude that the primary effect of hodydamycin is scission of DNA, followed by reduction of protein synthesis.

The present results are similar to those effects observed by YAMAKI *et al.*¹⁰⁾ and ONO *et al.*¹¹⁾ working with bleomycin and neocarzinostatin respectively.

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