ON THE MODE OF ACTION OF YEMENIMYCIN

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The effects of yemenimycin on the growth and contents of DNA, RNA and proteins of *Candida albicans* were appraised. Glycolysis and aerobic respiration were less sensetive to the antibiotic than the syntheses of nucleic acids and proteins. Incorporation of P-32 in DNA and RNA was markedly arrested by yemenimycin whereas the contents of the labeled supplement in TCA soluble fraction and phospholipids were appreciably augmented. Yemenimycin restricted the incorporation of C^{14}-thymidine and to a lesser extent of C^{14}-uridine in *Bacillus subtilis* cells while the incorporation of H^3-lysine in cellular protein was relatively the most resistant. Synthesis of aminoacyl-tRNA and the binding of C^{14}-phenylalanyl-tRNA to ribosomes in response to poly U were found to be rather insensitive to the drug. Finally the effect of yemenimycin on the protein synthesis of intact rabbit reticulocytes and their cell-free system was assessed. The antibiotic could influence unfavorably the synthesis of protein.

Yemenimycin was first isolated by SHIMI *et al.*[1] from cultures of *Streptomyces* AS-Y-52 obtained from the soil of Yemen lands. The antibiotic possesses notable activities against fungi and gram-positive bacteria whereas it exhibits high toxicity towards mice.

The present paper describes the studies conducted to elucidate the mode of action of the antibiotic.

Experimental

Organisms and growth media :

The organisms used were *Candida albicans* AS and *Bacillus subtilis* AA. The first organism thrives well on HAYDUCK and SABOURAUD media while the bacterium grows on a medium of the following composition (g/liter): glucose 10, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 2.5, $(NH_4)_2SO_4$ 1, $FeSO_4 \cdot 7H_2O$ 0.002, $MnCl_2 \cdot 4H_2O$ 0.002 and tryptone 1.

Viable count :

Cultures of *C. albicans* grown overnight were harvested, washed and rasuspended in fresh medium. Shaking was resumed for two hours, and the antibiotic was then supplemented at the MIC level (0.012 mcg/ml). After four hours of incubation

Table 1. Cell replication of *Candida albicans* in presence of yemenimycin

Medium	Dose (mcg/ml)	Incubation period (hrs.)	Viable cell number/ml
SABOURAUD	0	0	4×10^6
	0	4	6×10^8
	(MIC) 0.012	4	4×10^6
	(MIC×10) 0.12	4	2×10^5

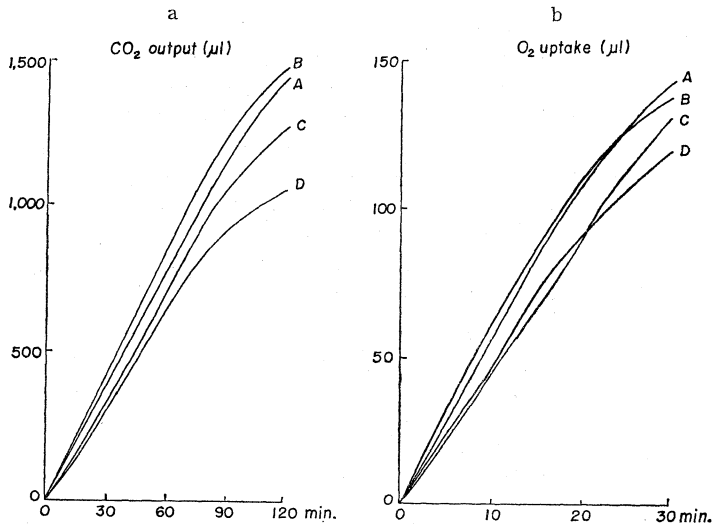
with shaking, aliquots were withdrawn, diluted with sterile saline solution which were then plated on SABOURAUD's agar and the number of colonies that developed after 24 hours were counted (Table 1).

Effects of yemenimycin on glycolysis and respiration :

To assess the glycolytic activities of *C. albicans*, a conventional WARBURG's manometric technique was applied[2]. Cells were grown overnight, washed with sterile saline solution and then suspended in M/15 phosphate buffer of pH 7.0. The reaction flask included 1.0 ml of the suspension which contained *ca.* 14 mg of cellular material and the antibiotic. The total volume was made up to 3.0 ml with the buffer. Glucose was used as substrate at final concentration of 0.02 mg/ml. Incubation temperature was 30°C and the gas phase was pure nitrogen. Carbon dioxide evolution was measured at 10-minute intervals (Fig. 1a). Respiration was measured by determining the uptake of oxygen using glucose as substrate (Fig. 1b).

Fig. 1

A : Control C : 0.012 mcg/ml (MIC)
B : 0.0012 mcg/ml D : 0.12 mcg/ml

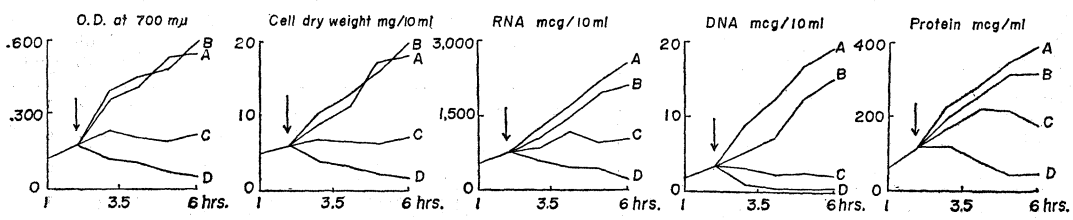


Effect on the growth and on contents of nucleic acids and protein :

Candida cells were grown overnight in SABOURAUD's medium, then collected, washed and finally suspended in 20 volumes of fresh medium. The cells were allowed to develop for two hours, the antibiotic was subsequently added and shaking was then resumed. Samples were withdrawn at different time intervals and assessed for their optical density at 700 mμ, cell dry weight and their contents of RNA, DNA and proteins after fractionation by the method of SCHMIDT-THANNHAUSER[3]. Contents of RNA were assayed by the orcinol method[4], of DNA by the indol method[5] and of proteins by FOLLIN's method[6] (Figs. 2 a~e).

Fig. 2

A : Control B : 0.0012 mcg/ml C : 0.012 mcg/ml (MIC) D : 0.12 mcg/ml



Incorporation of P-32 phosphoric acid into *Candida* cells :

Cells at early logarithmic phase were used (*ca.* 2~3 mg dry weight/ml of medium). Carrier free P-32 phosphoric acid was added to the medium at a final concentration

Table 2. Effect of yemenimycin on incorporation of P-32 phosphoric acid in cellular components of *C. albicans*

Incubation periods (min.)	Dose (mcg/ml)	Phospholipids		Acid soluble		RNA		DNA	
		c.p.m/mg cells dry wt.	% Incorporation	c.p.m/mg cells dry wt.	% Incorporation	c.p.m/mg cells dry wt.	% Incorporation	c.p.m/mg cells dry wt.	% Incorporation
30	0	3,154	100	3,921	100	1,897	100	441	100
	0.0012	3,532	112	4,103	105	1,822	96	327	74
	0.012	3,601	114	4,310	110	1,560	82	287	65
	0.12	3,436	109	4,372	111	1,421	75	169	38
60	0	3,561	100	4,157	100	2,567	100	649	100
	0.0012	4,277	120	4,519	109	2,421	94	428	66
	0.012	4,595	129	4,891	118	1,873	73	305	47
	0.12	3,772	106	4,902	118	1,781	69	207	32
90	0	4,012	100	4,213	100	3,071	100	879	100
	0.0012	4,768	119	4,720	112	2,930	95	502	57
	0.012	4,479	112	4,973	118	1,906	62	395	37
	0.12	4,011	100	4,517	107	1,562	51	246	28

Fig. 3-a

The *B. subtilis* AA cells collected at their exceptional growth phase, resuspended in the fresh medium containing 0.5 μCi C^{14}-uridine and 25 μCi H^3-lysine in 12.5 ml liquid that gave turbidity of 0.1 at 660 mμ. Incubation was resumed at 37C for 10 minutes, then to half of the fermentation medium dioxane solution of yemenimycin was added to attain the MIC level. Samples were withdrawn at different incubation intervals and incorporation of radioactive precursors was determinad by liquid scintillation spectrometer with conventional toluene scintillation mixture.

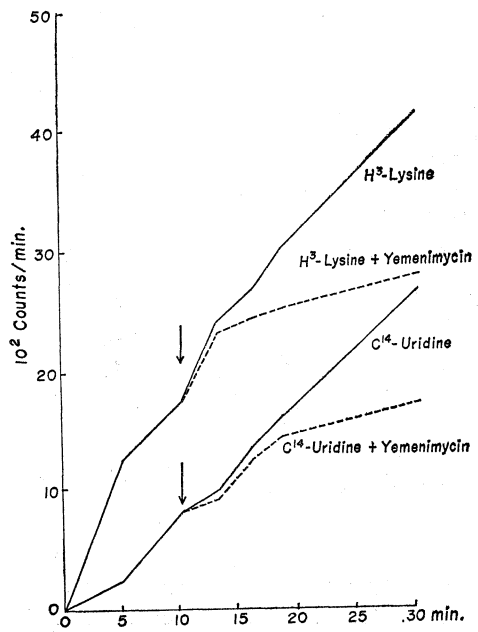


Fig. 3-b

Experimental conditions were the same as described in legend to Fig. 3-a except that C^{14}-thymidine (5 μCi) ; H^3-lysine (25 μCi) and deoxyadenosine 2.5 mg existed instead of the radioactive precursors used in experiment cited in Fig. 3-a.

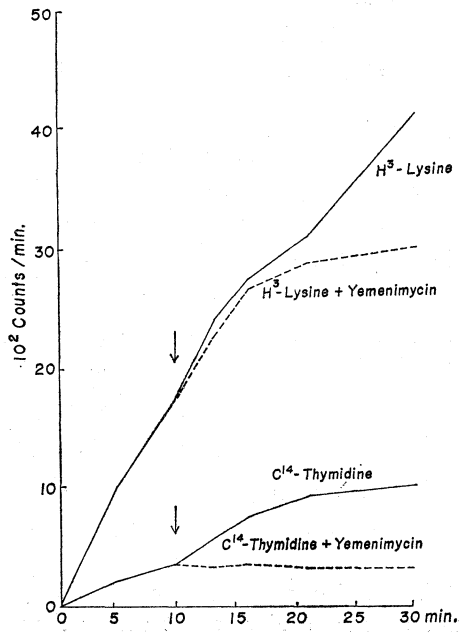


Table 3. Effect of yemenimycin on aminoacyl-tRNA synthesis

Concentration of yemenimycin (mcg/ml)	C^{14}-Aminoacids rendered TCA insoluble					
	Incubation period at 37°C					
	5 min.		10 min.		20 min.	
	c.p.m	% Incorporation	c.p.m	% Incorporation	c.p.m	% Incorporation
0.0	7,891	100	9,416	100	10,635	100
0.05	8,059	102	9,623	102	10,689	101
0.50	7,883	100	8,960	93	10,576	99
5.00	8,071	102	9,481	101	10,487	98

Reaction mixture (in 0.2 ml) : 0.2 mg tRNA ; 0.2 mg protein of S 100 fraction ; 0.4 μmoles ATP ; 10 μmoles Tris (pH 7.8) ; 20 μmoles KCl ; 2 μmoles $MgCl_2$ and 0.04 μCi C^{14}-aminoacid mixture.
Radioactivity was counted by a windowless gass flow counter.
Protein hydrolysate from *Chlorella* : Specific activity 48.2 mCi/milliatom of carbon.

of 0.5 μc/ml. Phospholipids, TCA soluble, RNA and DNA fractions were obtained by the method of KATCHMAN and FETTY[7] and then counted in a liquid scintillation spectrometer with a convential toluene scintilation mixture (Table 2).

The effect of yemenimycin at the MIC level (0.8 mcg/ml) on the incorporation of H^3-lysine, C^{14}-uridine and C^{14}-thymidine in cellular constituents of *B. subtilis* AA was appraised after TANAKA *et al.*[8] (Figs. 3 a, b)

Table 4. Effect of yemenimycin on binding of aminoacyl-tRNA to ribosomes

Reaction mixture		C^{14}-Phenylalanyl-tRNA bound to ribosomes	
		c.p.m	% Incorporation
Complete		896	100
−Poly U		48	
+Yemenimycin	0.05 mcg/ml	935	104
	0.50	872	97
	5.0	916	102
+Tetracycline	100.0	262	29

Complete reaction mixture (in 0.2 ml) : 0.33 mg ribosomes ; 10 mcg poly U ; 44 mcg C^{14}-phenylalanyl-tRNA ; 10 μmoles Tris (pH 7.6) ; 20 μmoles KCl and 4 μmoles $MgCl_2$.
Incubation at 30°C for 10 minutes.
Radioactivity was assessed by the windowless gass flow counter.

Synthesis of aminoacyl-tRNA in presence of the antibiotic was studied after TANAKA *et al.*[9] and FUJIMOTO *et al.*[10]; collecting tRNA precipitated with 5% TCA on millipore filter. Details are given in Table 3.

The method of NIRENBERG and MATTHAEI[11] was applied for examining the binding of C^{14}-phenylalanyl-tRNA ; prepared by the method of NATHANS and LIPMANN[12]; to ribosomes in response to poly U. Details are given in legends of Table 4.

Finally, the effect of the antibiotic on protein synthesis of intact reticulocytes and their cell-free system was studied. The cells were prepared by the method of ALLEN and SCHWEET[13] while their lysate was prepared after NISHIMURA[14] (Tables 5 and 6).

The chemicals used throughout the present study were all of the purest grade while the labeled compounds were the products of Amersham/Searle, Illinois, U.S.A.; Radiochemical Centre, Amersham, England and Schwartz Biological Research Inc.—Orangeburg-U. S. A. Poly U was produced by Miles Laboratories Inc.

Discussion

The number of colonies of *C. albicans* were markedly reduced by yemenimycin which seemed to exert a cidal effect rather than a static one. The lowest level of the antibiotic

had a detectable stimulatory effect on CO_2 output. A similar effect could be observed on the oxygen uptake at the early stages of the experiment. Higher levels of yemenimycin induced a slight decrease in the rates of respiration and glycolysis which still continued at considerably high rates. Except for the lowest concentration of yemenimycin the antibiotic influenced unfavorably the growth of *C. albicans*. Contents of DNA were the most sensitive to the drug while those of protein were relatively the least affected. The effects of the antibiotic intensified by progressing periods of incubation. Yemenimycin stimulated the incorporation of P-32 labeled supplement in TCA soluble fraction and in phospholipids whereas it reduced considerably the labeling of DNA and to a lesser extent to that of RNA of the *Candida* cells.

Table 5. Effect of yemenimycin on protein synthesis of intact reticulocytes

Concentration of antibiotic (mcg/ml)		Incorporation of C^{14}-valine			
		10 min.		20 min.	
		c. p. m	% Incorporation	c. p. m	% Incorporation
Control		2,237	100	5,129	100
Yemenimycin	1.0	2,314	103	4,988	97
	3.0	2,012	90	4,003	78
	5.0	1,788	80	3,087	60
Chloramphenicol	20.0	1,815	81	4,267	83

Incubation at 37℃.
Reaction mixture (in 0.5 ml) : 0.4 ml cell suspension containing 1.68 mg protein ; 0.025 μCi C^{14}-valine (7.2 mCi/mM) and the antibiotic.
Incorporation of C^{14}-valine into hot 5% TCA insoluble fraction was assessed by windowless gass flow counter.

Table 6. Effect of yemenimycin on protein synthesis of cell-free system of reticulocytes

Concentration of antibiotic (mcg/ml)		Incorporation of C^{14}-valine			
		10 min.		20 min.	
		c. p. m	% Incorporation	c. p. m	% Incorporation
Control		983	100	1,392	100
Yemenimycin	1.0	995	101	1,367	98
	3.0	898	91	1,112	80
	5.0	809	82	895	64
Chloramphenicol	20.0	843	83	1,178	85

Incubation at 37℃.
Reaction mixture/ml : 0.6 ml lysate (3.2 mg protein) ; 300 mcg tRNA ; 1 μmoles ATP ; 1 μmoles GTP ; 0.4 μmoles phosphoenol pyruvate ; 50 mcg pyruvate kinase ; 1 μmoles DTT ; 30 μmoles Tris-HCl of pH 7.8 ; 50 μmoles KCl ; 4 μmoles $MgCl_2$ and 0.4 μCi C^{14}-valine.
Radioactivity was determined by the windowless gass flow counter.

In general, the antibiotic exerted a weak unfavorable influence on glycolysis and respiration of the *Candida* cells. The lowest level of yemenimycin could stimulate glycolysis and the early stages of respiration whereas it hindered appreciably the biosynthesis of nucleic acids and protein. This could possibly indicate that neither respiration nor glycolysis were the primary site of action of the drug. Distribution of P-32 among DNA, RNA, TCA soluble and phospholipid fractions demonstrated the high susceptibility of DNA synthesis to the antibiotic. Relatively high labeling in the TCA soluble fraction would not be unexpected when nucleic acid biosynthesis was partially arrested. In view of these findings it is feasible to assume that yemenimycin could initially inhibit the biosynthesis of DNA in the *Candida* cells and that other metabolic disturbancies occurred as secondary consequences.

The incorporation of H^3-lysine into *B. subtilis* cells was found to continue after cessation of thymidine incorporation which occurred almost instantaniously after the addition of the antibiotic. The uridine incorporation was relatively less sensitive to the drug than that of thymidine but still more susceptible when compared with the lysine. The synthesis of aminoacyl-tRNA and the binding of C^{14}-phenylalanyl-tRNA to ribosomes in response to poly U were rather insensitive to yemenimycin. This combined information would indicate that the antibiotic interfered preferentially in the synthesis of DNA which, when arrested, would influence unfavorably other dependant metabolic activities.

Finally the results concerning the protein synthesis in reticulocytes and their cell-free

system were, however, anticipated. Arrestation of the protein synthesis induced by yemenimycin would probably occur as a secondary consequence to an initial inhibition of DNA synthesis.

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