CYCLAMIDOMYCIN (DESDANINE*), AN INHIBITOR OF NUCLEOSIDE DIPHOSPHOKINASE OF *ESCHERICHIA COLI*

TETSUJI SAEKI, MAKOTO HORI and HAMAO UMEZAWA

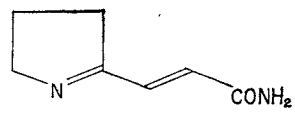
Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

(Received for publication April 8, 1972)

Cyclamidomycin (CAM) inhibited the growth of *E. coli* in a progressive manner even at high concentrations and the effect was abolished by washing the cells in a CAM-free medium. Among cellular macromolecular syntheses, nucleic acid synthesis was most sensitive to CAM. In the presence of CAM, nucleic acid precursors in the cellular pool were not incorporated into nucleic acids but were degraded and released into the medium. The nucleoside diphosphokinase activity of the 100,000×*g* supernatant from *E. coli* was inhibited by CAM, leading to the conclusion that this enzyme is involved in the mechanism of action of this antibiotic.

Cyclamidomycin (CAM) was isolated from cultures of a streptomyces, No. MA130-A1, by TAKAHASHI *et al.*[1] and shown to be identical to pyracrimycin A which had been reported earlier by CORONELLI *et al.*[2] Its structure is given in Fig. 1. We have studied the mode of action of CAM using *Escherichia coli* as a test organism and found that it is a specific inhibitor of nucleoside diphosphokinase (EC 2.7.4.6) of *E.coli*.

Fig. 1. Structure of cyclamidomycin
trans 3-(1-Pyrrolin-2-yl) acrylamide



Experimental results in support of this conclusion are presented in this paper.

Materials and Methods

Microorganisms and media: *E. coli* B was used throughout the *in vivo* experiments. Culture medium (CG-M) contained in 1,000 ml, 10 g of casamino acids, 2 g of glucose, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2$, 2 g of KH_2PO_4, 7.3 g of Tris (hydroxymethyl)-aminomethane and sufficient amount of conc. HCl to bring the final pH to 7.4.

Measurememt of cell growth: *E. coli* B was grown at 37°C with shaking in set of two L-tubes, each containing 9.5 ml of CG-M. Growth was followed by reading the optical density at 600 mμ with a Coleman spectrophotometer. When the O.D. of the cultures reached 0.20, one tube received 500 μg of CAM dissolved in 0.5 ml of water while the other received 0.5 ml of plain water. Incubation and absorbancy readings were continued.

Measurement of the number of viable cells: Cells were grown and CAM was added as above. At the indicated times, 0.1 ml samples were taken and serially diluted with 0.85% NaCl solution. The numbers of viable cells in the diluted suspensions were determined by the colony counting method (spread plate method on nutrient agar plates, 9 cm diameter).

Measurement of cellular macromolecule synthesis: Cells were grown in CG-M as indicated above. When cultures reached 0.20 O.D. at 600 mμ, 0.45 ml aliquots were dis-

* see Addedum

tributed into 3 sets of 2 tubes under ice cold conditions. In each set, one tube received one of the following radioactive precursors which had been dissolved in 50 μl of water, while the other tube received the same precursor and 25 μg of CAM which had been dissolved in 50 μl of water. For determination of protein synthesis, cell wall synthesis, and nucleic acid synthesis, 5 μCi/tube of ^{14}C-isoleucine, 0.3 μCi/tube of ^{14}C-N-acetylglucosamine and 0.5 μCi/tube of ^{14}C-adenine was used, respectively. These assay mixtures were incubated at 37°C, and at indicated intervals, 0.1 ml samples of the cell suspension were removed and placed on Whatman 3-mm paper discs. These were immersed in ice-cold 5% TCA solution for 30 minutes, and then washed twice with 5% TCA, once with ethanol and twice with ether. After drying, the discs were assayed for radioactivity in a Beckman liquid scintillation counter with the dioxan-scintillation system.

Mesurement of the incorporation of ^{14}C-adenine into various cellular fractions: Cells were grown as above until 0.20 O.D. at 600 mμ was reached. Then one tube received 10 μCi of ^{14}C-adenine and 500 μg of CAM which had been dissolved in 0.5 ml of H_2O, while the other received the radioactive precursor alone in the same manner. Incubation was reinitiated and at 2 minutes intervals, 0.5 ml samples were withdrawn from each tube, mixed with 3 volumes of unlabelled cell suspension (10 mg wet cells/ml), washed twice by centrifugation and suspension with ice-cold CG-M containing unlabelled adenine. Each cell pellet was twice extracted with 2 ml of cold 5% perchloric acid to obtain the acid soluble fraction and from the resulting precipitate, the DNA and RNA fractions were prepared by the method of SCHMIDT and THANNHAUSER[3]. The radioactivity in each fraction was measured in a Beckman liquid scintillation counter.

Labelling cells with ^{32}Pi and chase: Cells were grown as above except shat KH_2PO_4 in CG-M was reduced to 1/10 of the usual amount while 100 μCi of ^{32}Pi was added. When cultures reached an O.D. of 0.30 (600 mμ), cells were harvested, washed twice with CG-M and suspended in sufficient CG-M to obtain the original cell density. The suspension was divided into two 4.5 ml portions, one received 250 μg of CAM in 0.5 ml of CG-M, while the other received 0.5 ml CG-M. Incubations were started again and at times indicated, 0.5 ml samples were removed, chilled rapidly, and centrifuged at 4°C to separate cells from the medium. The acid-soluble (nucleotide pool) and the acid-insoluble fractions of the cells were then isolated.

DNA synehesis in toluenized *E. coli* cells: *E. coli* W 3110 polA$^-$ cells were toluenized and DNA synthesis in such cells were determined by the method of KOHIYAMA and KOLBER[4].

Enzyme preparation and assay conditions: (1) RNA polymerase (EC 2.7.7.6) was a DEAE-cellulose fraction prepared from *E. coli* Q 13 by the method of BERGESS[5] and was assayed according to his method except that the incubation period was limited to 20 minutes. (2) Nucleotide kinase (EC 2.7.4.4) and nucleoside diphosphokinase were prepared and their activities were assayed as follows: *E. coli* B was grown to late log phase in 10 SAKAGUCHI flasks (125 ml/flask) at 37°C with reciprocal shaking. The constituents of the medium were as follows: K_2HPO_4, 1.1%; KH_2PO_4, 0.85%; yeast extract, 0.6%; glucose, 1% and water. Cells were collected by centrifugation at 4°C and washed with 3 volumes of cold 5% NaCl - 0.5% KCl solution. Approximately 6 g of cell paste was obtained per liter of culture. Cells were disrupted and the 105,000$\times g$ (for 1 hour) supernatant fraction was prepared for assay of both enzyme activities. Throughout these procedures the method of SUGINO *et al.*[6,7] was employed unless otherwise indicated. ^{3}H-AMP and ^{3}H-CDP were the substrates for nucleotide kinase and nucleoside diphosphokinase, respectively, and unless otherwise stated, both assays were conducted identically. The enzyme reactions were terminated by heating at 100°C for 3 minutes and then a 10 μl aliquot of the heated solution and 10 μl of the carrier solution (containing AMP, ADP and ATP for nucleotide kinase and CMP, CDP and CTP for nucleoside diphosphokinase) were applied to a polyethylenimine-cellulose (PEI-cellulose) sheet. The chromatograms were developed in a stepwise fashion as follows: to 2.5 cm with 0.5 M formate buffer pH 3.4, to 7 cm with the same

buffer (2 M) and to 15 cm with the same buffer (4 M). Nucleotides were detected with an UV lamp. Appropriate spots were cut out and their radioactivities were measured in a Beckman scintillation counter with the toluene-scintillation system.

Sources of chemicals: CAM was a gift of Dr. S. TAKAHASHI of this Institute; the labelled nucleotides, New England Nuclear; the other labelled compounds, Dai-ichi Chemical Co.; the unlabelled, nucleotides, Boeringer Mannheim; calf thymus DNA, Worthington-Biochemical; PEI-cellulose sheet, J. T. Baker Chemical Co.

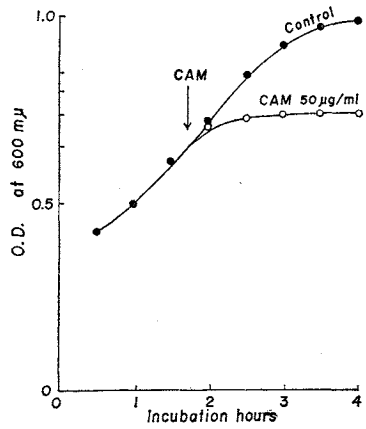
Results

Effects of CAM on Cell Growth and Cell Viability

Cell growth, followed either by determining the turbidity of the culture or counting cells in suspension, was inhibited in a progressive manner by 50 μg/ml of CAM, with a period of 30~60 minutes required for complete arrest. After complete inhibition of growth, the cells resumed exponential growth with only a short delay if they were freed from CAM by centrifugation and then resuspended in fresh medium. These results are shown in Figs. 2 and 3, and indicate that CAM acts reversibly, being bacteriostatic rather than bacteriocidal.

Fig. 2. Effect of CAM on the growth of *E. coli* B

CAM was added at the time indicated and growth was followed as described in "Materials and Methods".



Effect of CAM on the Syntheses of Cellular Macromolecules

To determine whether CAM preferentially inhibits synthesis of nucleic acids, proteins, or cell walls, *E. coli* were labelled, in the presence or absence of 50 μg/ml CAM, with ^{14}C-adenine, ^{14}C-isoleucine, and ^{14}C-N-acetylglucosamine, respectively. As shown in Fig. 4, the synthesis of nucleic acid was inhibited most rapidly and strongly. At 8 minutes after addition of CAM, nucleic acid synthesis was inhibited by 76% whereas protein

Fig. 3. Effect of CAM on cell viability of *E. coli* B

CAM was added at time 'Zero' and subsequently the numbers of viable cells were determined at the times indicated as described in "Materials and Methods".

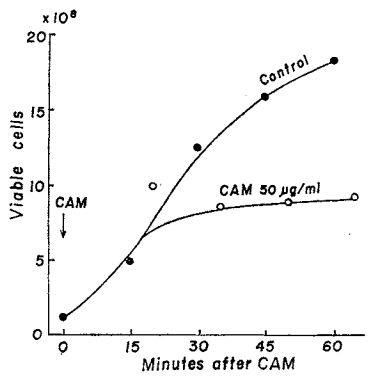


Fig. 4. Effect of CAM on the synthesis of cellular macromolecules

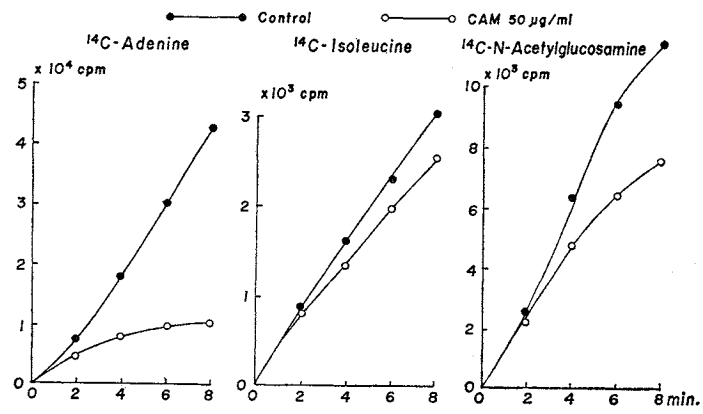


Table 1. Effect of CAM on the incorporation of ^{14}C-adenine into various cellular fractions of *E. coli* B

Minutes after addition of CAM	Incorporation as % of control		
	Nucleotide pool	RNA	DNA
6	37	10	2
10	20	5	1

The concentration of CAM was 50 μg/ml and the results were expressed as per cent of control. Details are described under "Materials and Methods".

Table 2. Effect of CAM on RNA polymerase reaction

		^{14}C-AMP incorporated into acid ppt	
		cpm	% of control
Complete		7,223	100
+CAM	15 (μg/ml)	4,838	67
	30	4,214	58
	60	3,878	54
+Rifampicin	40	82	1
+Actinomycin D	40	853	12
−Enzyme		18	0.2
−DNA		211	1.4

The reaction mixture contained in 0.25 ml ; 10 μmoles of Tris-HCl (for final pH 7.9), 2.5 μmoles of $MgCl_2$, 25 mμmoles of EDTA, 25 mμmoles of dithiothreitol, 37.5 μmoles of KCl, 10 mμmoles of K_2HPO_4, 0.1 μmole of CTP, UTP and CTP, 0.1 μCi of ^{14}C-ATP, 78 μg of calf thymus DNA, 50 μl of enzyme solution and 10 μl of drug solution (containing an indicated drug at the desired concentration). Mixtures were incubated for 20 minutes at 37℃, then chilled and 5 ml of 5% TCA was added. After 30 minutes the precipitates were collected on Millipore filters and washed with cold 5% TCA. After drying, radioactivity on the filters was determined in a Beckman liquid scintillation counter with a toluene scintillation system.

Table 3. Effect of CAM on DNA synthesis in toluene-treated *E. coli* cells

	^{3}H-TMP incorporated into acid ppt	
	cpm	% of control
Complete	1,333	100
+CAM 50 (μg/ml)	1,315	99
100	1,244	93
+NEM 1.5 mM	148	11
−3 dXTP	0	0
−ATP	254	19

Preparation of toluenized cells and assay conditions are described under "Materials and Methods". A reaction mixture, 0.15 ml, consisted of the following components : 50 mM of Tris buffer (pH 7.4), 5 mM of $MgCl_2$, 90 mM of KCl, 1 mM of mercaptoethanol, 1 μM of tRNA's (*E. coli*), 35 μM of 4 dNTPs, 1 mM of ATP, 1.5 μCi/ml of ^{3}H-TTP and a desired amount of drug. The reaction was started by addition of 25 μl of the suspension of toluenized *E. coli* W 3110 polA$^-$ (about 1.6×10^8 cells) to the remainder which had been warmed at 37℃. The incubation was carried out without shaking at the same temperature for 30 minutes and terminated by addition of 0.15 ml of 1 M NaOH−0.02 M EDTA. The mixture was heated for 30 sec. at 100℃, cooled to 0℃ and 0.15 ml of 1M HCl was added. One half ml of 10% ice-cold TCA was added and the precipitates were collected on Whatman glass fiber papers, rinsed with 10 ml ice-cold 0.5% TCA and then with 10 ml of ice cold ethanol. The papers were dried and counted in a Beckmann liquid scintillation counter with a toluene scintillation system.

and cell wall syntheses were inhibited only by 16% and 34%, respectively. However the latter two syntheses also ceased by 20 minutes.

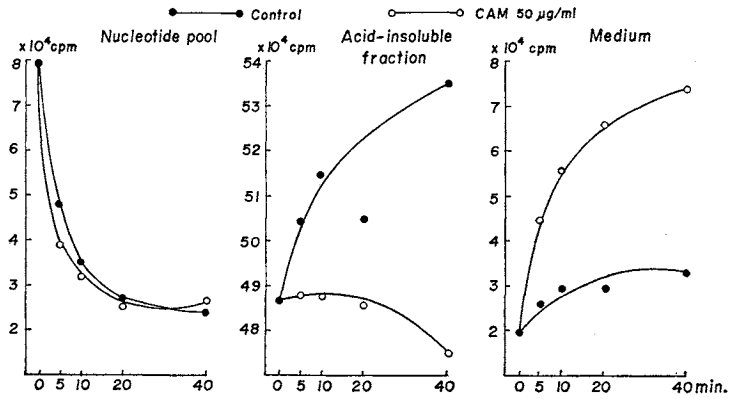
Both DNA and RNA syntheses were similarly inhibited (Table 1). Since CAM inhibited DNA and RNA syntheses to a similar extent, the following sites of action were considered. (1) CAM could bind to DNA template, resulting in inhibition of both replication and transcription ; (2) CAM could inhibit an early step in nucleic acid metabolism, such as membrane transport of a precursor or nucleotide interconversion. These possibilities were tested.

Does CAM Interact with DNA?

The RNA polymerase reaction is reported to be most sensitive to those compounds which bind to DNA and affect its structure and template function[8]. As shown in Table 2, CAM inhibited the reaction to some extent. However, in a separate experiment, CAM did not change the Tm of calf thymus DNA (the result is not given). In addition, as shown in Table 3, CAM also did not inhibit DNA synthesis in toluenized *E. coli* cells. These results suggested that CAM does not interact with DNA.

Furthermore, considering that both DNA and RNA syntheses *in vivo* are completely inhibited in the presence of 50 μg/ml of CAM (Table 1), the partial inhibition of RNA polymerase (Table 2) may be regarded as a minor effect.

Fig. 5. Effect of CAM on the fate of cells prelabelled with ^{32}Pi
The ordinate represents the counts in 0.5 ml aliquots of the cell suspension. Details are dercribed under "Materials and Methods".



Effect of CAM on the Fate of Nucleotide Pool

By a label-chase experiment, the flow of radioactivity from the nucleotide pool to nucleic acid was followed in the presence or absence of CAM (Fig. 5). In control cells, radioactivity disappeared from the nucleotide pool and appeared in the nucleic acid fraction. In the cells exposed to CAM, the radioactivity of the nucleotide pool was not incorporated into the nucleic acid fraction but was released into the medium. An analogous experiment using ^{14}C-adenine-labelled cells showed that CAM induced the release of adenine and adenosine into medium (results are not shown). These observations, together with the preceding results (Table 1 and Fig. 4), strongly suggested that CAM acts by reducing the availability of substrates for DNA and RNA syntheses.

Effect of CAM on Nucleotide Kinase and Nucleoside Diphosphokinase from *E. coli*

These enzymatic steps are closely concerned with the availability of nucleoside triphosphates for nucleic acid synthesis. Nucleotide kinase, which catalyzed the

Table 4. Effect of CAM on adenylate-kinase of *E. coli* B

	AMP kinase activity	
	cpm	% of control
Complete	2,230	100
+CAM 20 (μg/ml)	2,262	101
40	2,156	97
80	2,161	97
−$MgCl_2$	100	4
−ATP	82	4

A reaction mixture contained in 0.25 ml ; 10 μmoles of Tris-HCl (pH 7.5), 1.0 μmole of ATP, 2.0 μmoles of $MgCl_2$, 0.1 μmole of ^3H-ATP (0.5 μCi/μmole), various amounts of CAM and 50 μl of enzyme solution (106 μg protein/ml). After incubation for 20 minutes at 37℃, the reaction was stopped by heating at 100℃ for 3 minutes. The chromatograms were prepared as described in "Materials and Methods", and the enzyme activity is expressed as the total counts incorporated into both ADP and ATP spots.

Table 5. Inhibition of nucleoside diphosphokinase by CAM

	NDP kinase activity	
	cpm	% of control
Complete	1,256	100
+CAM 20 (μg/ml)	679	63
40	333	31
80	150	13
160	87	7

Reaction mixtures were prepared as described in the legend to Table 4 except that each contained 0.1 μmole of ^3H-CDP (2.5 μCi/μmole) and 50 μl of enzyme solution (17.5 μg protein/ml) instead of ^{14}C-AMP. After incubation for 10 minutes at 37℃, the reaction was stopped by heating (100℃ for 3 minutes).

The chromotograms were prepared as described in "Materials and Methods" and the enzyme activity is expressed as the counts located in the CTP spot.

conversion of nucleoside monophosphate to diphosphate has specificity for bases but not for the sugar moiety (ribose or deoxyribose)[6]. In *E. coli*, 5 distinct nucleotide kinases differing in specificity were reported to be present[6]. On the other hand, nucleoside diphosphokinase, which catalyzes the conversion of nucleoside diphosphate to triphosphate, has no specificity either for bases or the sugar moiety[9]. Adenylate kinase (EC 2.7.4.3) was arbitrarily chosen from the nucleotide kinases and its possible sensitivity to CAM was determined. As shown in Table 4, this enzyme was only slightly inhibited by CAM even at 80 μg/ml. Other nucleotide kinases have not yet been tested. Table 5 shows a marked inhibition of nucleoside diphosphokinase by CAM. At a concentration of as low as 20 μg/ml, CAM exerted an obvious inhibition (63 % of control). Kinetic studies are in progress using a purified enzyme preparation.

Discussion

The observation that CAM is a potent inhibitor of nucleoside diphosphokinase (Table 5) provides a reasonable explanation for the simultaneous and progressive inhibition of DNA and RNA syntheses. The antibiotic could reduce the availability of all the nucleoside triphosphates by inhibiting this enzyme.

Since protein synthesis and cell wall synthesis are also dependent on the supply of nucleoside triphosphates they are eventually inhibited by CAM. However, we cannot account for the considerable delay with which the syntheses of these macromolecules were inhibited. The simplest explanation would be that under normal conditions the supply of nucleoside triphosphate is not as rate-limiting for these syntheses as it is for nucleic acid synthesis. Also, we do not know at present why the nucleotide pool becomes unstable (Fig. 5) in the presence of CAM. Catabolic enzymes may somehow become induced or activated by CAM. These points are currently under investigation.

Acknowledgement

The authors are grateful to Dr. MARCO RABINOVITZ, National Institutes of Health, Bethesda, for proof-reading and criticism.

Addendum

After this paper was submitted, identity of desdanine, pyracrimycin A, and cyclamidomycin was established[10]. REUSSER has reported[11] recently that desdanine preferentially inhibited oxidative phosphorylation in rat liver mitochondria. Study on nucleoside diphosphokinase of bacterial origin may provide further insight into the mechanism of oxidative phosphorylation in mitochondria.

References

1) TAKAHASHI, S.; M. NAKAJIMA, Y. IKEDA, S. KONDO, M. HAMADA, K. MAEDA & H. UMEZAWA: A new antibiotic, cyclamidomycin. J. Antibiotics 24 : 902~903, 1971
2) CORONELLI, C.; G. TOMONI, G. BERETTA & G. C. LANCINI: Production, isolation and properties of pyracrimycin A. J. Antibiotics 24 : 491~496, 1971
CORONELLI, C.; A. VIGEVANI, B. CAVALLER & G. G. CALLO: Structure determination of pyracrimycin A. J. Antibiotics 24 : 497~502, 1971
3) SCHMIDT, G. & S. T. THANNHAUSER: A method for the detection of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. 161 : 83~89, 1945
4) KOHIYAMA, M. & A. R. KOLBER: Temperature sensitive mutant of the DNA replication system in *Escherichia coli*. Nature 228 : 1157~1160, 1970
5) BERGESS, R. R.: A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. 244 : 6160~6167, 1969
6) HIRAGA, S. & Y. SUGINO: Nucleotide monophosphokinase of *Escherichia coli* infected and uninfected with an RNA phage. Biochim. Biophys. Acta 114 : 416~418, 1966.

7) SUGINO, Y.; H. TERAOKA & H. SHIMONO: Metabolism of deoxyribonucleotides. I. Purification and properties of deoxycytidine monophosphokinase of calf thymus. J. Biol. Chem. 241 : 961～969, 1966
8) KAGEYAMA, M.; M. HASEGAWA, A. INAGAKI & F. EGAMI: Interaction of antibiotics with deoxyribonucleic acids. J. Biochem. 67 : 549～557, 1970
9) KOERNER, J. F.: Enzymes of nucleic acid metabolism. Ann. Rev. Biochem. 39 : 291～322, 1970
10) ARGOUDELIS, A.D.; H. HOEKSEMA & H.A. WHALEY: Identity of desdanine, pyracrimycin A and cyclamidomycin. J. Antibiotics (in press)
11) REUSSER, F.: Desdanine, an inhibitor of oxidative phosphorylation in mitochondria. Canad. J. Microbiol. 18 : 265～269, 1972