Communication to the Editor

BIOLOGICAL EFFECTS OF AZOMYCIN (2-NITRO-IMIDAZOLE): INHIBITION OF RIBONUCLEOTIDE REDUCTASE

Sir:

Azomycin was isolated from culture filtrates of *Nocardia mesenterica* by MAEDA *et al.*[1] in 1953 and identified as 2-nitro-imidazole by NAKAMURA *et al.*[2] in 1955.

It is active against gram-positive bacteria including mycobacteria, gram-negative bacteria and protozoa. We have studied its biochemical effect mostly on *Bacillus subtilis* and *Escherichia coli* and found that ribonucleotide reductase is a site of action of azomycin. Several lines of evidence leading to this conclusion are as follows: The effect of azomycin on cellular macromolecule synthesis was determined by use of *B. subtilis* as a test organism. Protein synthesis, nucleic acid synthesis and cell wall synthesis were followed by determining incorporation of ^{3}H-leucine, ^{14}C-adenine and ^{14}C-N-acetylglucosamine into acid-insoluble materials of cells, respectively. As shown in Fig. 1, the quickest and most marked response to azomycin was observed with ^{14}C-adenine incorporation. As early as 5 minutes after addition of azomycin, the incorporation of ^{14}C-adenine was inhibited by 57 % as opposed to a rather slower effect on the other parameters. Since a separate experiment revealed that azomycin did not cause degradation of RNA and DNA *in vivo*, the above inhibition of adenine uptake should be regarded as direct inhibition of nucleic acid synthesis but not the result of stimulated break-down. Rapid inhibition of nucleic acid synthesis was also observed with *E. coli*. In order to obtain further insight into the inhibition of nucleic acid synthesis, cells were labelled with ^{14}C-adenine in the presence or absence of azomycin and after various incubation periods,

Fig. 1. Effect of azomycin on the syntheses of cellular macromolecules

B. subtilis was grown at 37°C with shaking in 3 sets of duplicate L-tubes. All tubes contained 9.3 ml of a culture medium which contained in 1,000 ml; 5 g of glutamic acid, 2 g of glucose, 3 g of NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2$, 2 g of KH_2PO_4 and 7.3 g of tris (hydroxymethyl)-aminomethane, pH 7.4. When the cell density reached 0.1 O.D. at 600 nm, each tube received one of the following radioactive precursors which had been dissolved in 0.5 ml of water and one minute later, one of each set of 2 tubes received 200 μg of azomycin which had been dissolved in 0.2 ml of water (plus azomycin run), while the other received 0.2 ml of water (control run). For determination of nucleic acid synthesis, protein synthesis and cell wall synthesis, 1 μCi/tube of ^{14}C-adenine (2 mCi/m mole), 2 μCi/tube of ^{3}H-leucine (4 mCi/m mole) and 0.34 μCi/tube of ^{14}C-N-acetylglucosamine (3.4 mCi/m mole) were used, as pecursors, respectively. At indicated intervals, 0.5 ml samples were withdrawn from each tube and mixed with 5 ml of 5 % trichloroacetic acid (TCA) solution in an ice bath. Acid insoluble materials were collected on Millipore membrane filter disks (pore size 0.45 μ) and washed twice with 5 ml of cold 5 % TCA solution. After drying, the fiters were determined for radioactivity in a Beckman liquid scientillation counter with the toluene-scintillation system.

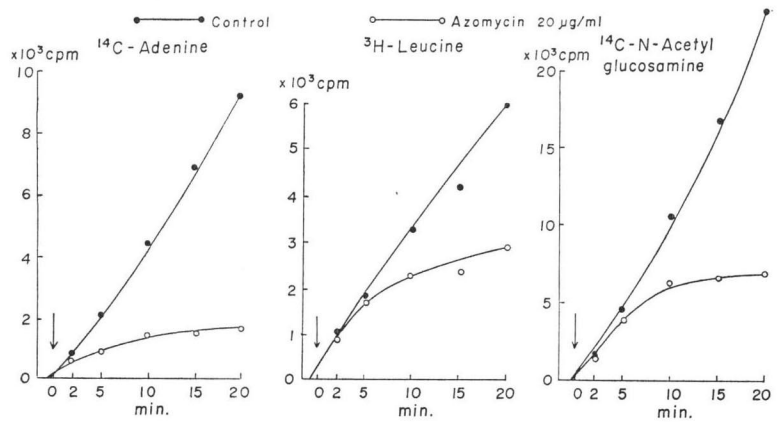

Table 1. Effect of azomycin on the incorporation of ^{14}C-adenine into various cellular fractions

B. subtilis was grown in an L-tube containing 10 ml of the culture medium as described in the legend to Fig. 1. When the culture reached the cell density of 0.1 O.D. at 600 nm, the cell suspension was divided into two 4.5 ml portions, one received 100 μg of azomycin and 2 μCi of ^{14}C-adenine (plus azomycin run), which had been dissolved in 0.5 ml of water, while the other received the radioactive precursor alone (control run) in the same manner. At 3-minute 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 culture medium containing unlabelled adenine. Each cell pellet was extracted twice with 2 ml of 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 THANHAUSER[3]. The radioactivity in each fraction was measured in a Beckman liquid scintillation counter.

Minutes after addition of azomycin	cpm/sample					
	Nucleotide pool		RNA		DNA	
	control	+azomycin	control	+azomycin	control	+azomycin
3	896	968 (108)*	5,332	3,436 (64)	180	100 (56)
6	1,992	1,012 (51)	12,130	6,322 (52)	409	122 (30)
12	4,868	2,336 (48)	30,018	14,836 (49)	1,382	262 (19)

* (% of control)

cell samples were withdrawn with which radioactivities in the nucleotide pool and the DNA and RNA fractions were determined. As shown in Table 1, the strongest inhibition was observed with the incorporation of ^{14}C-adenine into the DNA fraction throughout the incubation period. These results suggested that azomycin primarily interferes with a reaction(s) which is involved in DNA synthesis. Antibiotics which bind to the DNA template, covalently or noncovalently, tend to inhibit DNA and RNA syntheses and this possibility was tested. Azomycin showed no effect on DNA synthesis in toluene-treated *E. coli* cells.[7] Therefore, it was thought azomycin does not inhibit polymerization step. Azomycin did not inhibit *in vitro* the RNA polymerase reaction, either. These observations strongly suggest that azomycin does not interact with DNA template. Based on these observations and considering the preferential inhibition of DNA synthesis *in vivo*, we suspected that azomycin possibly inhibits ribonucleotide reductase, the enzyme catalyzing the step where the biosynthetic route of deoxyribonucleotides branches from that of ribonucleotides. In *E. coli*, all four ribonucleoside diphosphates, namely, ADP, GDP, UDP, and CDP are directly reduced to the corresponding deoxy analogs by ribonucleotide reductase. As shown in Table 2, the enzyme from *E. coli* was sensitive to azomycin and the rate of inhibition was a function of azomycin concentrations ranging from 25 μg/ml to 100 μg/ml. The results presented so far led us to conclude that ribonucleotide reductase is a major site of action of azomycin. However, the bactericidal effect of the antibiotic, as described below, can not be explained by the above mechanism of action alone. If cells of *B. subtilis*, growing exponentially, were exposed to 20 μg/ml of azomycin for 10 minutes, viable number of cells decreased to 1/100. Bactericidal agents should cause irreversible effect on cell components of central importance, such as cell membranes, the DNA template, the enzymes involved in RNA and protein syntheses, ribosomes and *etc.* For example, streptomycin which irreversibly inactivates ribosomes could be bactericidal because production of new ribosomes requires the function of unimpaired ribosomes *per se*. Irreversible damage on cell membranes may also lead to cell death because reproduction of cell membranes needs transport of essential molecules. Assuming that azomycin irreversibly modifies ribonucleotide reductase, the effect alone should not be lethal to the organism, since the enzyme can be newly synthesized once the antibiotic is removed by washing or dilution. Azomycin did not inhibit polyU-directed polyphenylalanine synthesis with *E. coli* ribosomes at concentrations as high as 100 μg/ml, suggesting

no interaction with ribosomes.

Therefore, the bactericidal effect of azomycin seems to remain unanswered.

Table 2. Inhibition of ribonucleotide reductase by azomycin

Enzyme solution was prepared from *E. coli* Q 13 by the method of REICHARD *et al.*[4] Reaction mixture contained in a volume of 1.02 ml; 2 μmoles of ATP, 15 μmoles of $MgCl_2$, 1 μmole of TPNH, 2 μmoles of sodium pyrophosphate, 0.2 μCi of ^{14}C-CMP, 0.5 ml of enzyme solution and a desired amount of azomycin. Mixtures were incubated for 30 minutes at 37°C, and chilled. To each chilled mixture, 3 ml of methanol was added and mixed. After standing for 3 hours in an ice bath, the mixtures were centrifuged and each supernatant fluid was taken and evaporated to dryness *in vacuo* and the residue was hydrolyzed in 1 ml of 1.2 N HCl at 95°C for 30 minutes. After HCl was exhaustively evaporated off *in vacuo*, the residue was dissolved in 0.1 ml of water containing unlabelled CMP and dCMP. A 20 μl sample of the solution was applied to a sheet of polyethylenimine-cellulose and chromatographed with the solvent system according to RANDERATH[5] (6 g of $Na_2B_4O_7 \cdot 10H_2O$, 3 g of H_3BO_3 and 25 ml of ethylene glycol in 70 ml of water). Nucleotides were detected with a UV lamp. Appropriate spots were cut out and their radioactivities were measured in a Beckman liquid scintillation counter with the toluene-scintillation system. The assay was conducted under conditions where the rate of reaction was a linear function of incubation time and enzyme amount. For the full enzyme activity, all ATP, Mg^{++}, TPNH and sodium pyrophosphate were required[4,6].

Azomycin μg/ml	dCMP formed in 30 min.	
	cpm/mg protein	% of control
None (control)	1,229	100
25	946	77
50	795	65
100	283	23

Acknowledgement

We thanks Mr. KAZUHIRO TANAKA for his techcical assistance.

TETSUJI SAEKI
HAMAO UMEZAWA
Institute of Microbial Chemistry
Kamiosaki, Shinagawa-ku, Tokyo, Japan
TAEKO TOKIEDA-FUJISHIGE
MAKOTO HORI*
Showa College of Pharmaceutical Sciences
Tsurumaki, Setagaya-ku, Tokyo, Japan

(Received November 22, 1973)

References

1) MAEDA, K.; T. OSATO & H. UMEZAWA: A new antibiotic, azomycin. J. Antibiotics, Ser. A 6: 182, 1953
2) NAKAMURA, S. & H. UMEZAWA: Structure of azomycin (2-nitro-imidazole). J. Antibiotics, Ser. A 8: 66, 1955
3) SCHMIDT, G. & S. T. THANHAUSER: A method for the detection of desoxy-ribonucleic acid, ribonucleic acid and phosphoproteins in animal tissue. J. Biol. Chem. 161: 83~89, 1945
4) REICHARD, P.; A. BALDESTEN & L. RUTBERG: Formation of deoxycytidine phosphates from cytidine phosphates in extracts from *Escherichia coli*. J. Biol. Chem. 236: 1150~1157, 1961.
5) RANDERATH, K. & E. RANDERATH: Ion-exchange thin-layer chromatography. XIV. Separation of nucleotide sugars and necleoside monophosphates on PEI-cellulose. Anal. Biochem. 13: 575~579, 1965
6) BISWAS, C.; J. HARDY & W. S. BECK: Release of repressor control of ribonucleotide reductase by thymine starvation. J. Biol. Chem. 240: 3631~3640, 1965
7) KOHIYAMA, M. & A. R. KOLBER: Temperature sensitive mutant of the DNA replication system in *Escherichia coli*. Nature 228: 1157~1160, 1970

* To whom all correspondence should be directed.