# MECHANISM OF GROWTH INHIBITION BY 2′-AMINO-2′-DEOXYGUANOSINE IN *ESCHERICHIA COLI*

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The mechanism of inhibition of *Escherichia coli* by the new nucleoside antibiotic, 2′-amino-2′-deoxyguanosine (2AG), is described. Upon the addition of 2AG, the syntheses of macromolecules continued for 15 minutes. After this lag time, protein synthesis sharply decreased, RNA synthesis slightly decreased, but DNA synthesis was not affected. Tritiated 2AG was readily incorporated into the acid-soluble fraction of cells in the form of the mono-, di- and triphosphates. In the acid-soluble fraction, radioactivity was found only in the RNA fraction. The major part of the radioactivity was found to be guanylate; only 25% existed as the nucleotide of 2AG. In resistant strains of *E. coli*, there was a lower degree of phosphorylating activity and less incorporation of 2AG into RNA per unit of cell mass increase. These results suggest that 2AG inhibits growth by its incorporation into RNA and the subsequent disturbance of RNA function causing a block in protein synthesis.

We reported in the preceding paper[1] that a novel nucleoside antibiotic, 2′-amino-2′-deoxyguanosine (2AG)[2,3] produced by *Enterobacter cloacae*[4], was lethal to some strains of *Escherichia coli*. The antibacterial activity of 2AG was reversed by guanosine and other purine nucleosides. 2AG incorporation into the cell differed from that of guanosine and guanosine non-competitively inhibited the uptake of 2AG. Furthermore, we showed that differences in sensitivity to 2AG among *E. coli* strains does not depend upon the ability to take up 2AG into the cell.

The action mechanisms of many purine nucleoside antibiotics have been classified into three main categories[5]; inhibition of biosynthesis of precursors of macromolecules, inhibition of macromolecular synthesis, and inhibition of RNA or DNA function by incorporation into the polynucleotide. We now report on the inhibition of protein synthesis by 2AG and the mechanism of resistance to 2AG.

## Materials and Methods

Microorganisms and culture conditions. *Escherichia coli* KY 3591, KY 3591 2AG$^r$ and K-12 described in our previous report[1] were grown in the media shown in Table 1. All cultures were incubated at 30°C with shaking. Growth was measured as described previously[1].

Paper and thin-layer chromatography. Ascending paper chromatography was carried out with Toyo Roshi No. 51A filter paper. Cellulose thin-layer chromatography was done with Avicel SF (Funakoshi).

The following solvent systems were used:

Solvent A: *iso*-butyric acid – acetic acid – 1 N ammonia (10: 1: 5)
B: *n*-butanol – acetic acid – water (4: 1: 2)
C: water (pH 10)
D: 80% formic acid – *n*-butanol – *n*-propanol – acetone – 30% trichloroacetic acid (5: 8: 4: 5: 3)

For the separation of nucleotides, polyethylene-imine cellulose thin-layer chromatography (PEI-

Table 1. Composition of media

| | Medium B | Medium N | Medium P |
|---|---|---|---|
| $KH_2PO_4$ | 13.6 g/liter | 13.6 g/liter | 13.6 g/liter |
| $(NH_4)_2SO_4$ | 2.0 g/liter | 2.0 g/liter | 2.0 g/liter |
| Glucose | 3.0 g/liter | 3.0 g/liter | 3.0 g/liter |
| $MgSO_4$ | 95 mg/liter | 95 mg/liter | 95 mg/liter |
| $FeSO_4 \cdot 7H_2O$ | 0.5 mg/liter | 0.5 mg/liter | 0.5 mg/liter |
| Thiamine·HCl | 0.1 mg/liter | 0.1 mg/liter | 0.1 mg/liter |
| Casamino acid | 3.0 g/liter | 1.5 g/liter | 0.5 g/liter |
| Uracil | — | 10 mg/liter | 10 mg/liter |
| Thymine | — | 1 mg/liter | — |

Before autoclaving, pH was adjusted to 7.0 with KOH.

TLC) was carried out with Polygram CEL 300PEI (Brinkmann Instrument Inc.) using 0.75 M $KH_2PO_4$ as solvent.

Measurement of radioactivity. Radioactivity was measured as described previously[1].

Macromolecular syntheses. The syntheses of DNA, RNA and protein were followed by measuring the incorporation of labeled thymine (methyl-$^3$H), uracil-2-$^{14}$C and L-leucine-4,5-$^3$H into acid-insoluble precipitates. After the addition of radioactive precursors, 0.5 ml samples were removed at intervals, and poured into 2.5 ml of ice-cold 5% trichloroacetic acid (TCA), and placed for one hour in the ice bath. They were filtered through HA Millipore filters (0.45 $\mu$) and washed with 15 ml of cold 5% TCA. The filters were dried and counted in vials containing toluene scintillation fluid.

Fractionation of macromolecules. A modified SCHMIDT-THANHAUSER-SCHNEIDER method[6] was used for the fractionation of macromolecules. The lipid fraction was extracted with ethanol - ether (1: 1). The RNA fraction was decomposed by incubation with 0.3 N potassium hydroxide for 18 hours at 37°C. From the acid-insoluble fraction of the residue, the DNA fraction was solubilized in 5% perchloric acid by heating for 15 minutes at 100°C. The residue after these treatments was taken as the protein fraction.

Phosphatase treatment. Acid phosphatase (625 units/mg protein) was prepared from *Penicillium decumbens*. An aqueous sample was added to an equal volume of enzyme solution (2 mg protein/ml of 0.2 M acetate buffer (pH 4.0) and incubated for 2 hours at 37°C.

Chemicals. The radioactive compounds were purchased as follows: thymine(methyl-$^3$H) (20 Ci/m mole) and L-leucine-4,5-$^3$H (58 Ci/m mole) from Radiochemical Centre, Amersham; uracil-2-$^{14}$C (53.2 Ci/mole) from Daiichi Pure Chemicals. Tritiated 2AG (2AG-8-$^3$H) (6.4 Ci/mole) was prepared as described previously[1]. Guanine nucleotides were the products of our company. Phosphorylated 2AG (2AG-5′-phosphate) was prepared chemically by us (unpublished data).

## Results

### Effects of 2AG on the Syntheses of Cellular Macromolecules in *E. coli* KY 3591

The effects of 2AG on the syntheses of DNA, RNA and protein in growing cells are presented in Fig. 1. Inhibition by 2AG of DNA synthesis was only slight and detected only after 60 minutes. However, RNA synthesis was inhibited at 15~30 minutes and stopped at about 40 minutes. Protein synthesis was blocked at about 15 minutes. These results indicated that 2AG primarily inhibits protein synthesis, and has some effect on RNA synthesis, without affecting DNA synthesis.

The inhibition of protein synthesis by chloramphenicol occurred in five minutes after addition (data not shown). The different lag time between 2AG and chloramphenicol suggests that 2AG must be metabolized to the actual inhibitor or that 2AG only indirectly inhibits protein synthesis.

### Distribution of 2AG-8-$^3$H in Cells

Fig. 1. Effects of 2AG on macromolecular syntheses in *E. coli* KY 3591

(A) Incorporation of thymine-$^{3}$H and uracil-$^{14}$C into the acid-insoluble fraction.

When the cell density of *E. coli* KY 3591 in medium N reached $OD_{660nm}=0.1$, thymine(methyl-$^{3}$H) (1 $\mu$Ci/ml) and uracil-2-$^{14}$C (0.2 $\mu$Ci/ml) were added. After incubation for 30 minutes, it was divided into 2 portions and 2AG (10 $\mu$g/ml) was added to one. After the addition of radioactive precursors, 0.5 ml samples were removed at intervals and treated as described in Materials and Methods.

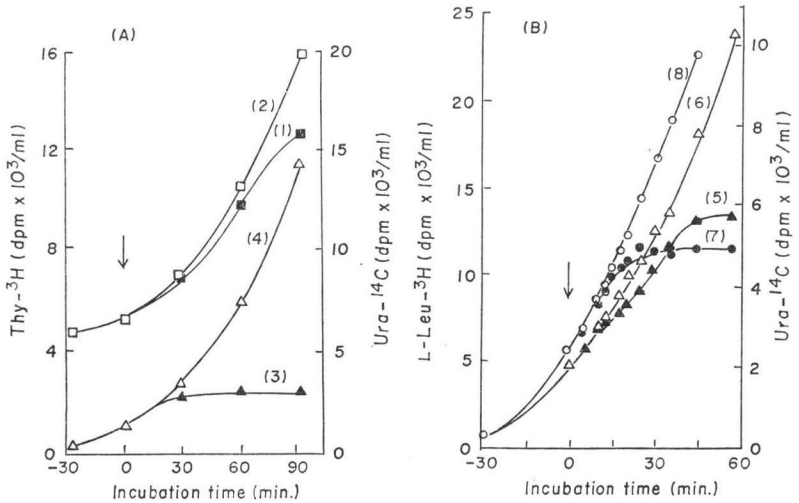
Incorporation of thymine-$^{3}$H in the presence (1) or absence of 2AG (2). Incorporation of uracil-$^{14}$C in the presence (3) or absence of 2AG (4).

(B) Incorporation of uracil-$^{14}$C and L-leucine-$^{3}$H into acid-insoluble fraction.

Experimental procedures were the same as those described in (A) except that medium P and L-leucine-4,5-$^{3}$H were used instead of medium N and thymine-$^{3}$H.

Incorporation of uracil-$^{14}$C in the presence (5) or absence of 2AG (6). Incorporation of L-leucine-$^{3}$H in the presence (7) or absence of 2AG (8).

The arrows indicate the time of 2AG addition.




When exponentially growing culture was incubated with 2AG-8-$^{3}$H, the radioactivity was linearly incorporated into the acid-soluble fraction from 5 minutes to 90 minutes. After incubation for 45 minutes, the cells were collected by centrifugation and fractionated as described in Materials and Methods. The results are shown in Table 2. Most of the acid-insoluble radioactivity was found in the RNA fraction (94.5%).

As shown in Fig. 2, the paper chromatogram of the acid-soluble fraction showed that the radioactivity was found at the spot corresponding to 2AG and between Rf 0.3~0.6. By treatment of the acid-soluble fraction with acid phosphatase, the radioactivity at Rf 0.3~0.6 disappeared and that corresponding to 2AG increased. It was confirmed by paper chromato-

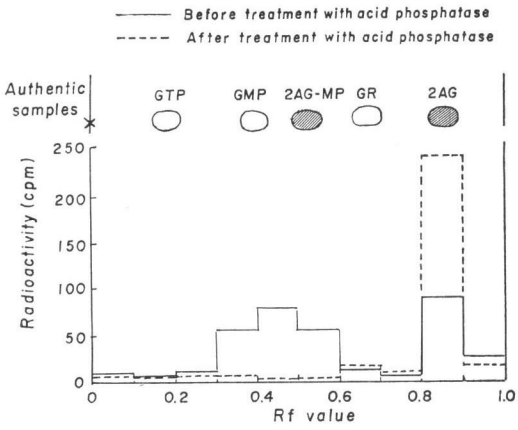
Table 2. Distribution of 2AG-8-$^{3}$H incorporated into *E. coli* cells

The incubation of *E. coli* KY 3591 was performed using a 300 ml baffled Erlenmeyer flask containing 40 ml of medium B. The culture was grown to $OD_{660nm}=0.2$ (*ca* $5\times10^{8}$ cells/ml), 2AG-8-$^{3}$H was added as 1.0 $\mu$Ci/10$\mu$g/ml and incubation was continued for 45 minutes. The bacterial cells were collected by centrifugation (0°C, 5,000×*g*, 5 minutes) and were fractionated by a modified SCHMIDT-THANNHAUSER-SCHNEIDER method as described in Materials and Methods.

| Fractions | Total counts (cpm) | Percent |
|---|---|---|
| Cells | $1.01\times10^{6}$ | |
| Acid-soluble | $8.70\times10^{5}$ | 86 |
| Acid-insoluble | $1.43\times10^{5}$ | 14 |
| DNA | $4.30\times10^{3}$ | 3.0 |
| RNA | $1.35\times10^{5}$ | 94.5 |
| Protein | $1.60\times10^{3}$ | 1.1 |
| Lipid | $1.90\times10^{3}$ | 1.3 |

Fig. 2. Paper chromatogram of acid-soluble fraction of *E. coli* cells incubated with 2AG-8-$^3$H

The acid-soluble fraction was obtained as described in Table 2. As the solvent system for paper chromatography, *iso*-butyric acid - acetic acid - 1 N ammonia (10: 1: 5) was used. The spots of authentic samples were detected by UV-absorbance. The radioactivity of each fraction was determined after chopping the paper. The treatment with acid phosphatase was carried out in 0.1 M acetate buffer (pH 4.0) for 2 hours at 37°C.




graphy with two solvent systems (B and D) that this latter spot was 2AG. These results suggest that the substance(s) at Rf 0.3~0.6 was not a degradation product but a phosphorylated derivative(s) of 2AG.

The degree of phosphorylation was examined by ion-exchange cellulose thin-layer chromatography (PEI-TLC). From the Rf value of 2AG, it was expected that phosphorylated 2AG would show a higher Rf value than the corresponding phosphorylated guanosine because of the 2′-amino group (Fig. 3). Subsequently, it was confirmed that the spots of Rf 0.2, 0.4 and 0.65 were 2AG-triphosphate (2AG-TP), 2AG-diphosphate (2AG-DP) and 2AG-MP, respectively. The comparison of the chromatogram of the sensitive strain's acid-soluble fraction with that of the resistant strain is shown in Fig. 3. The resistant strain contained less 2AG-TP and 2AG-DP than did the sensitive strain. Furthermore, the incorporation of radioactivity into acid-insoluble fraction per unit of cell mass increase was larger in the sensitive strain than in the resistant strains by 2~2.5 times (Table 3). These observations suggest that weak 2AG-MP phosphorylating activity is one of the reasons for resistance to 2AG.

Fig. 3. Polyethylene-imine cellulose thin-layer chromatogram of acid-soluble fraction of *E. coli* cells incubated with 2AG-8-$^3$H

The acid-soluble fraction was obtained as described in Table 2. As the solvent, 0.75 M $KH_2PO_4$ was used. The spots of authentic samples were detected by UV-absorbance. The radioactivity of each fractions was determined after scraping the cellulose.

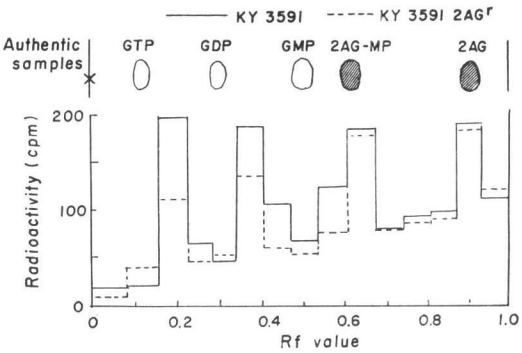



Table 3. Specific incorporation of 2AG-8-$^3$H into acid-insoluble fraction of various strains

Incubation was performed using large test tubes containing 5 ml of medium B. Exponentially growing cultures of *E. coli* KY 3591, KY 3591 2AG$^r$ and K-12 were adjusted to $OD_{660\,nm}=0.05$ with fresh medium and 2AG-8-$^3$H (0.18 $\mu$Ci/10 $\mu$g/ml) was added. After one hour incubation, 0.1 ml sample was removed and was poured into 0.5 ml of ice-cold 5% TCA and placed for one hour in the ice bath. Samples were then filtered as described in Materials and Methods. Strain K-12 is naturally resistant to 2AG.

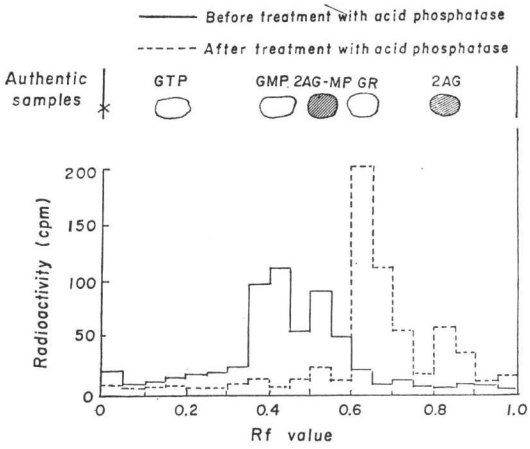
| Strains | $OD_{660\,nm}$ | | cpm/ml | cpm/ml / $\Delta OD=0.1$ |
|---|---|---|---|---|
| | 0 hour | 1 hour | | |
| KY 3591 | 0.050 | 0.065 | 32,100 | 214,000 |
| KY 3591 2AG$^r$ | 0.050 | 0.105 | 46,400 | 84,400 |
| K-12 | 0.050 | 0.115 | 47,200 | 72,600 |

Radioactive Substances in RNA Fraction

The paper chromatogram of the RNA fraction after alkaline hydrolysis is shown in Fig. 4. The main spots were identified as GMP and 2AG-MP. The chromatogram of the hydrolysate treated with acid phosphatase showed that only 25% of radioactivity was recovered as 2AG and the spot of major radioactivity migrated like guanosine. This latter substance was confirmed as guanosine by paper chromatography with two solvents (B and D). We could not detect any difference in the ratio of 2AG and guanosine between the sensitive and the resistant strain.

Fig. 4. Paper chromatogram of alkaline hydrolysate of acid-insoluble fraction

Experimental conditions were the same as those described in Fig. 2.




## Discussion

The antibiotic 2AG primarily inhibited protein synthesis in cells of *E. coli* KY 3591. The added 2AG was rapidly taken into the cells in a few minutes, but protein synthesis was not inhibited until about 15 minutes. In contrast, chloramphenicol, which is known to directly inhibit protein synthesis, blocked protein synthesis of strain KY 3591 in five minutes after its addition.

From above results, the following three inhibitory mechanisms of protein synthesis by 2AG could be suggested.

(1) 2AG-TP formed by a kinase in the cell acts as an analog of GTP which is required for the initiation step of protein synthesis. Consequently, only this step is blocked and the its elongation reaction can continue for several minutes.

(2) The 2AG-TP acts as an analog of GTP which is required for the elongation step of protein synthesis, but it takes time to convert 2AG to 2AG-TP or to obtain a high enough concentration of 2AG-TP in the cell.

(3) The 2AG-TP is incorporated into messenger-, transfer- and ribosomal-RNA as an analog of GTP. Consequently, these RNAs cannot exhibit normal function, and then protein synthesis is inhibited.

At present, it is not clear which mechanism is correct. These questions will be answered by using 2AG-TP in a cell-free protein synthesis system. However, mechanism 3) is supported by following evidence; (1) 2AG-8-$^{3}$H was incorporated into RNA as 2AG, although the major radioactivity was converted to guanosine. (2) HOBBS *et al.*[7] reported that poly(2′-amino-2′-deoxyuridylic acid) and poly-(2′-amino-2′-deoxycytidylic acid) were stable to ribonucleases and showed other abnormal properties. The 2AG containing RNA could be expected to have an abnormal structure and properties. (3) The nucleoside antibiotics, toyocamycin[8] and 3′-amino-3′-deoxyadenosine[9] were reported to be incorporated into RNA, preventing the maturation of ribosomal RNA in mammalian cells. From the above evidence, we propose that the mode of action of 2AG involves its incorporation into RNA, and as the result of production of non-functional RNA, protein synthesis is prevented.

Mutants resistant to purine nucleoside antibiotics, tubercidine[10] or other adenosine analogs[11] were produced by the loss of adenosine kinase which is a component of uptake mechanism. In the case of 2AG, the transport of this compound into the resistant bacterial cell is similar to that of the sensitive cell[1]. Although deamination at the 2′-position of 2AG could be considered to be an inactivation mechanism in *E. coli*, the ratio of guanosine-$^{3}$H to 2AG-$^{3}$H in the RNA of the resistant strain did not differ significantly from that of the sensitive strain. The difference between sensitive and resistant strains was found in the phosphorylation of 2AG-MP and the incorporation of 2AG into RNA. These fact also support the mode of action of 2AG postulated above.

It is of interest that most of the 2AG-8-$^{3}$H incorporated into RNA was converted to guanosine-$^{3}$H, suggesting the presence of an enzyme that acts on 2AG in the RNA chain because guanosine-$^{3}$H or tritiated guanine nucleotides could not be detected in the acid-soluble fraction of the cell (Fig. 2). The degradation of 2AG in the cell is still uncertain, and particularly the mechanism of 2′-deamination of 2AG is unknown.

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