UPTAKE OF 2′-AMINO-2′-DEOXYGUANOSINE BY *ESCHERICHIA COLI* AND ITS COMPETITION BY GUANOSINE

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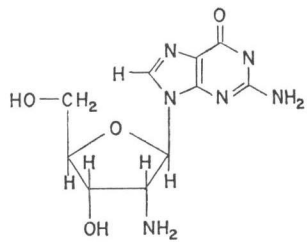
The antibacterial activity of a new nucleoside antibiotic, 2′-amino-2′-deoxyguanosine (2AG), is reversed by guanosine and other purine nucleosides. 2AG is apparently taken up by *E. coli* by a mechanism different from that of guanosine; guanosine inhibits this uptake non-competitively. Insensitive *E. coli* strains and the resistant mutant obtained from the sensitive strain also took up 2AG.

We reported previously[1,2,3] that a strain of *Enterobacter cloacae* produces a novel aminonucleoside antibiotic, 2′-amino-2′-deoxyguanosine (2AG), the structure of which is shown in Fig. 1. This compound inhibits only some strains of *Escherichia coli*, shows antitumor activity against sarcoma 180 in mouse, and is cytotoxic to HeLa cells.

Fig. 1. 2′-Amino-2′-deoxyguanosine (2AG)



Many nucleoside antibiotics have been described[4] and all such purine nucleosides are adenosine analogs. 2AG is the first guanosine analog and the first nucleoside containing 2-amino-2-deoxyribose found in nature. 2AG has been synthesized chemically by IKEHARA *et al.*[5] and HOBBS *et al.*[6]

In this paper, we describe the reversal of antibacterial activity of 2AG by guanosine, the inhibition of incorporation of 2AG into the cell by guanosine, and the lack of difference in uptake by sensitive and resistant strains.

Materials and Methods

Microorganisms. *Escherichia coli* KY 3591, K-12, KY 8232 and other strains were from our laboratory collection. Strain KY 3591 is the most sensitive to 2AG whereas strain K-12 is one of the insensitive strains. Strain KY 8232 is a purine-requiring mutant of K-12.

Media and culture conditions. Wild-type strains were grown in a medium having the following composition: KH_2PO_4, 13.6 g; $(NH_4)_2SO_4$, 2 g; glucose, 3 g; Casamino acids (Difco), 3 g; $MgCl_2$, 95 mg; $FeSO_4 \cdot 7H_2O$, 0.5 mg; thiamine-HCl, 0.1 mg per liter of water. For the growth of the purine-requiring strain, 100 mg of adenine per liter were added. All cultures were incubated at 30°C with shaking. For the agar diffusion assay of antibacterial activity, medium containing 0.8% agar was used and incubated at 30°C for 16 hours.

Determination of cell growth. The growth of cells was automatically recorded with Bio-photorecorder TN-112M (Toyo Kagaku Sangyo) or intermittently measured using a colorimeter (ANA-7A, Tokyo Koden) equipped with a red filter.

Paper chromatography. Ascending paper chromatography was carried out with Toyo Roshi

No. 51A filter paper. Water (pH 10) was used as solvent.

Measurement of guanosine and guanine. The concentration of guanosine and guanine in the medium was determined by scanning paper chromatogram with the Dual-Wavelength TLC Scanner CS 900 (Shimazu).

Measurement of radioactivity. The radioactivity of aqueous samples was determined in dioxane scintillation fluid containing 50 g of naphthalene, 10 g of 2,5-diphenyloxazole (PPO) and 0.25 g of 2,2′-*p*-phenylene-bis-(5-phenyloxazole) (POPOP) per liter of dioxane with a Packard Tricarb Scintillator. The radioactivity of Millipore filters was counted in toluene scintillation fluid containing 4 g of PPO and 0.1 g of POPOP per liter of toluene.

Incorporation of radioactive compounds into cells. After the addition of radioactive compounds, 0.5 ml samples were removed at intervals. They were immediately filtered through HA Millipore filters (0.45 μ) and washed with 15 ml of fresh medium without glucose. The filters were dried and counted in vials containing toluene scintillation fluid.

Preparation of 2AG-8-^{3}H. Preliminary experiment with deuterium oxide (D_2O). The method of EIDINOFF and KNOLL[7] was employed with some modifications. A preliminary experiment was carried out with D_2O in order to find a suitable condition for tritiation of 2AG. The reaction mixture contained 5 mg of reduced platinum, 0.5 ml of D_2O (99.75%) and 20 mg of 2AG. The following conditions were examined; (1) a tube was sealed immediately, (2) after freezing the reaction mixture, the tube was sealed off under vacuum, (3) after displacing the air by nitrogen gas, the tube was sealed. These sealed tubes were heated at 100°C for 18 hours. After the platinum was removed by centrifugation, deuterochloric acid was added to a concentration of 5% and the tubes were subjected to proton nuclear magnetic resonance spectroscopy in order to confirm the deuterium-substitution of the proton at position-8 of guanine. In no case was the signal of 8-^{1}H detected at 9.4 ppm, other proton signals were not affected by this treatment. These results showed that complete deuterium-substitution at 8-^{1}H was specifically obtained. When these reaction mixtures were developed with paper chromatography, some guanine was detected. No UV-absorbing spot was detected other than those of 2AG and guanine. The amount of guanine produced by side reactions under each condition was (1) 17.4%, (2) 13.5% and (3) 15.3%, respectively. These results showed that the different conditions affected the displacement reaction only very little. We adopted condition (3) for tritiation by considering the safety of the operation.

Tritiation of 2AG. Using the above condition, except for 0.5 ml of tritium oxide (0.5 Ci/ml) replacing D_2O, the tritiation reaction was carried out. After the reaction, 0.5 ml of 0.1 N hydrochloric acid was added and the supernatant solution was poured into 30 ml of acetone. The precipitates were washed with acetone twice. Dissolving the precipitates in one ml of 0.1 N hydrochloric acid, the same procedure as above was repeated twice in order to remove tritium oxide and exchangeable tritium in 2AG (*i.e.* $-O^{3}H$ and $-N^{3}H_2$). In order to eliminate degradation products, the reaction mixture was passed through the column of 10 ml of Dowex 50 × 8 (NH_4^+ form). Thirteen mg of 2AG were obtained with a specific activity of 6.4 mCi/m mole. No spot other than 2AG was detected by paper chromatography and the isotopic purity was confirmed.

Chemicals. 2′-Amino-2′-deoxyadenosine was obtained from Dr. M. IKEHARA of Osaka University. Guanosine-U-^{14}C (0.4 Ci/m mole) and tritium oxide (0.5 Ci/ml) were purchased from New England Nuclear. Purine related compounds, chloramphenicol, penicillin G, mitomycin C and 5-fluorouracil were products of Kyowa Hakko Kogyo Co. Ltd.

Results

Antibacterial Activity of 2AG

In a previous study, antibacterial activity of 2AG was detected only for one strain (KY 8323) of *E. coli* among three strains tested.[3] We further examined additional strains of *E. coli* in order to determine the antibacterial spectrum of 2AG. Among 20 strains tested, strain KY 3591 was the only strain sensitive to 2AG which gave a clear growth-inhibition zone. Other strains including K-12, B

Fig. 2. Growth of *E. coli* in the presence of 2AG

To exponentially growing cells of *E. coli*, 2AG was added at various concentrations. The incubation and measurement of bacterial growth were carried out in the Bio-photorecorder TN-112 (Toyo Kagaku Sangyo).

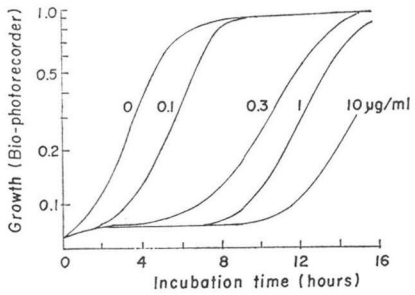


Fig. 4. Reversal of antibacterial activity of 2AG by purine related compounds

Incubation of *E. coli* KY 3591 was performed in the Bio-photorecorder. In the presence of 2AG (0.035 mM), purine related compounds were added at 0.35 mM.

1), without 2AG; 2), guanosine; 3), adenosine; 4), inosine; 5), deoxyguanosine; 6), deoxyadenosine; 7), 5′-adenylic acid; 8), 5′-guanylic acid; 9), none, guanine, adenine, hypoxanthine, xanthine, xanthosine, 5′-inosinic acid or 5′-xanthylic acid.

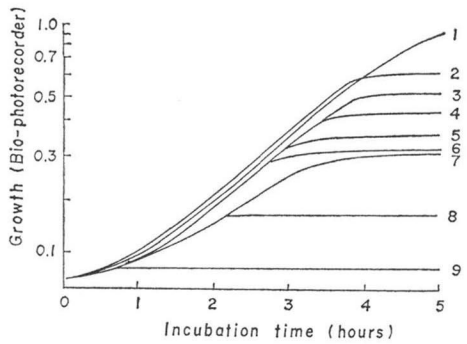
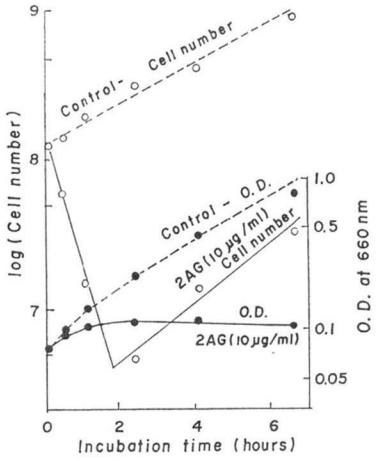


Fig. 3. Viability of *E. coli* KY 3591 after exposure to 2AG

The incubation of *E. coli* in the presence of 2AG (10 μg/ml) was performed in test tubes with shaking at 30°C. Growth was followed by increase in OD (Tokyo Koden Colorimeter with red filter). Viability of *E. coli* was measured by counting colonies on agar plates.



and a purine-requiring mutant of K-12 (KY 8232) were resistant to 2AG. Strain KY 3591 is a prototrophic strain showing growth on the minimum medium and its sensitivity to other drugs such as penicillin G, chloramphenicol, mitomycin C and 5-fluorouracil is similar to other strains. Since KY 3591 gave a clear growth-inhibition zone in contrast to the slightly turbid growth-inhibition zone of KY 8323, further tests were carried out with KY 3591.

The effect of 2AG on the growth of KY 3591 is shown in Fig. 2. Growth was delayed by concentration of 0.1 μg/ml and higher. The length of the lag before restoration of growth was dependent on the concentration of 2AG. The addition of more than 10 μg/ml did not further delay the restoration of growth. Organisms arising after prolonged culture in the presence of 10 μg/ml of 2AG were resistant to 2AG. As shown in Fig. 3, viable counts of KY 3591 dropped for 2 hours after addition of 10 μg/ml of 2AG, although the optical density of the culture remained unaltered after a slight increase in the first 0.5 hour. After 2 hours, viable counts increased due to the growth of resistant clones. From this culture, the resistant strain KY 3591 $2AG^r$ was isolated and used for further tests.

Chemically synthesized 2AG-5′-monophosphate (unpublished data) and 2′-amino-2′-deoxyadenosine[5] did not show any antibacterial activities.

Effects of Nucleic Acid Related Compounds on Antibacterial Activity of 2AG

Some nucleoside antibiotics are known to act as metabolic antagonists.[4] When the antibacterial activity of a metabolic antagonist is tested in the presence of a competitive metabolite by the agar diffusion method, the diameter of inhibition zone decreases according to the concentration of the competitive metabolite. When guanosine was added to the assay plates of 2AG, the diameter of the growth-inhibition zone did not decrease but the turbidity of the zone did. This result suggested that 2AG might not act as a metabolic antagonist of guanosine. Thus a detailed study was carried out on the effect of nucleic acid related compounds on the action of 2AG.

Fig. 5. Effect of concentration of guanosine on the antibacterial activity of 2AG

A) growth curves. In the presence of 2AG (10 μg/ml), guanosine was added at the concentrations indicated in the figure. Incubation was carried out in the Bio-photorecorder using the KY 3591 strain. The dotted curve shows the control experiment (without 2AG).

B) Relationship between the concentration of guanosine added to the medium and the level of cell growth after five hours.

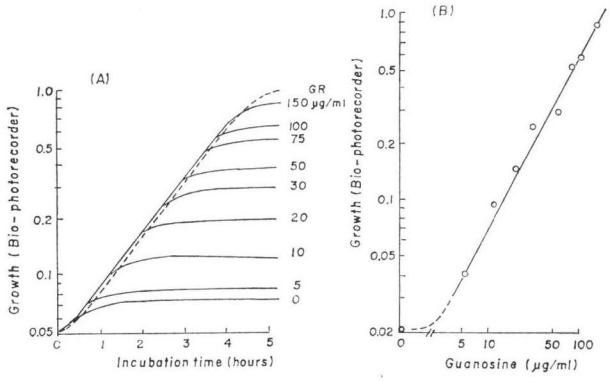


Fig. 6. Effect of addition time of guanosine on the restoration of growth of *E. coli* KY 3591

Guanosine was added at 100 μg/ml after incubation in the presence of 10 μg/ml of 2AG for various periods. The growth was followed with the Bio-photorecorder.

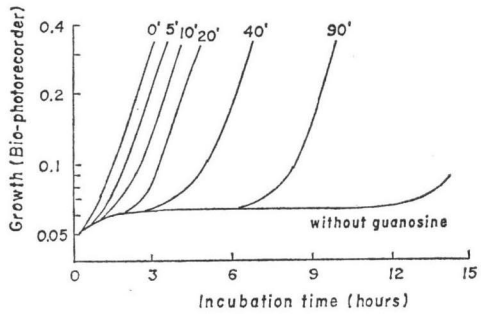
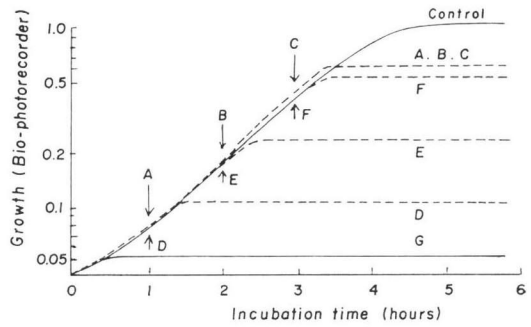


Fig. 7. The inhibitory action of 2AG on the growth of *E. coli* KY 3591 in the presence and absence of guanosine

In the presence of guanosine (100 μg/ml), 2AG was added at 1, 2 and 3 hours as indicated by arrows A, B and C, respectively. In the absence of guanosine, 2AG was added at the time of arrows D, E and F. Curve G shows the experiment in which 2AG was added at 0 time without guanosine. The growth was automatically recorded with the Bio-photorecorder.



As shown in Fig. 4, purine nucleosides except for xanthosine reversed growth inhibition by 2AG, although the degree of reversal differed depending on the nucleoside. AMP moderately reversed the action of 2AG, while GMP was weak. No activity was shown by IMP, XMP, xanthosine, purine bases, pyrimidine-related compounds, D-ribose, D-2-deoxyribose, D-ribose-5-phosphate, phosphoribosyl pyrophosphate, cyclic AMP and ppGpp. Furthermore it is of interest that the addition of purine

nucleosides did not affect the growth rate but only the extent of growth.

Effect of Guanosine on the Action of 2AG

Since guanosine was the most active agent for the reversal of growth inhibition by 2AG, a detailed study of the effects of guanosine was made. In the presence of 2AG (10 μg/ml), the final growth level increased according to the concentration of guanosine as shown in Fig. 5-A. When the logarithm of the growth level was plotted against the logarithm of concentration of guanosine, the growth level was proportional to the amount of added guanosine in the range of about 10~100 μg/ml

Fig. 8. The incorporation of 2AG-8-^{3}H and guanosine-U-^{14}C into *E. coli* KY 3591 cells

Guanosine and 2AG were added giving 0.04 μCi/50 μg/ml and 0.022 μCi/10 μg/ml at 0 hour, respectively.

The measurement of 2AG-^{3}H incorporation was described in Materials and Methods. The amount of guanosine-U-^{14}C and guanine-U-^{14}C in the supernatant of the medium was determined after separation by paper chromatography. The growth was measured with Tokyo Koden Colorimeter. (1), Cell growth in the presence of guanosine; (2), cell growth in the absence of guanosine; (3), guanosine-U-^{14}C in the supernatant of the medium; (4), guanine-U-^{14}C in the supernatant (the specific activity of guanine is equal to a half of guanosine in radioactivity because the guanine was uniformly labeled); (5), 2AG-8-^{3}H incorporated into cells in the presence of guanosine; (6), 2AG-8-^{3}H incorporated into cells in the absence of guanosine.

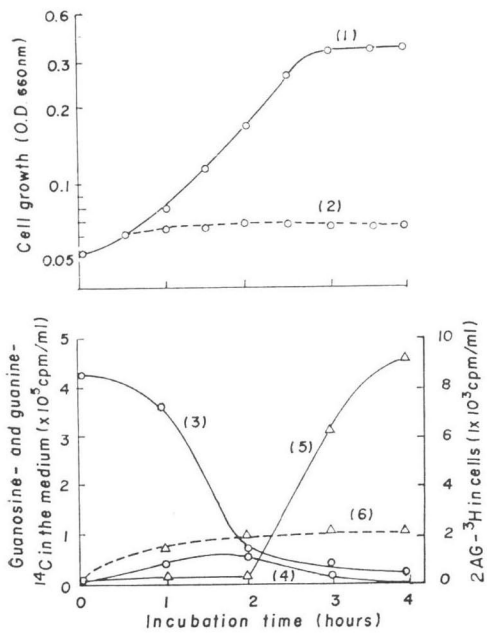


Fig. 9. Effect of guanosine concentration on the 2AG-uptake activity in *E. coli* KY 3591

Cell suspension of *E. coli* KY 3591 (10^8 cells/ml) in the medium was incubated at 30°C with 2AG-^{3}H and guanosine. The reciprocal of incorporation rate was plotted as a function of reciprocal of concentration of 2AG. The incorporation rate was expressed by the radioactivity incorporated per 10^8 cells in 20 minutes.

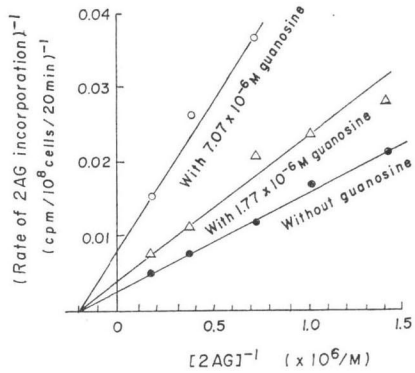
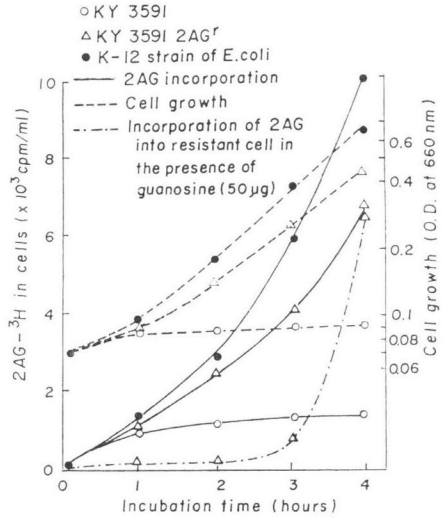


Fig. 10. The uptake of 2AG-8-^{3}H by 2AG resistant strains

To the exponentially growing cultures of *E. coli* strains, 2AG was added at 0.022 μg/10 μg/ml. The measurement of 2AG uptake is described in Materials and Methods.



as shown in Fig. 5-B. On the other hand, the effect of concentration of 2AG on the extent of growth was negligible in the presence of guanosine.

When the addition of guanosine was delayed in the presence of 2AG, the lag time for the restoration of growth was proportional to the delay time (Fig. 6). The time taken for growth to reach an OD of 0.2 was found to be 5 times the delay time. A similar phenomenon was found in an experiment in which washing was used for the elimination of 2AG instead of addition of guanosine (data not shown).

2AG (10 μg/ml) was added after incubation for various periods in the presence of guanosine (100 μg/ml) in order to see whether there is preferential uptake of 2AG. Cell growth consistently stopped at 3.5 hours of incubation despite changes in the addition time of 2AG; in the absence of guanosine, growth always stopped at 30 minutes after the addition of 2AG (Fig. 7). These results indicated that the presence of guanosine above a certain level prevents the uptake of 2AG.

Transport of 2AG and Guanosine into *E. coli*

The fate of added guanosine and 2AG in the medium was investigated by paper chromatography. As shown in Fig. 8, guanosine-U-^{14}C rapidly decreased to 20% of the initial amount within 2 hours and guanine increased to 25% of the initial amount of guanosine. This observation agreed with the result obtained by scanning of the paper chromatogram by UV absorption. The appearance of guanine in the medium suggests that guanosine was transported into the cell by the action of purine nucleoside phosphorylase.[8] Guanosine (50 μg/ml) disappeared after 2~3 hours and 100 μg/ml of guanosine disappeared by 3~4 hours. The uptake of guanosine was not affected by the addition of 2AG.

In the absence of guanosine, 2AG-8-^{3}H was immediately transported into the cells and the uptake ceased at about 1 hour because of the inhibition of cell growth. In the presence of 50 μg/ml of guanosine, the uptake of 2AG-8-^{3}H began only after incubation for 2 hours. This initiation point of uptake of 2AG coincided with the time of disappearance of most of the guanosine. The guanine produced in the medium did not appear to affect the transport of 2AG (see also Fig. 4). These results show that the uptake of 2AG was prevented by guanosine and only after the reduction of guanosine content was 2AG transported into the cell.

When 2AG-8-^{3}H was taken up by the cells, guanine-8-^{3}H was not detected in the medium; this was clearly different from the case of uptake of guanosine.

Kinetics of Transport of 2AG

Tritiated 2AG was taken up linearly into the cell for the first 30 minutes. Because of the low specific radioactivity of 2AG-^{3}H, the amounts of radioactivity taken up in 20 minutes was used as the rate of uptake. A LINEWEAVER-BURK plot of uptake of 2AG is shown in Fig. 9. From this figure, Km and V_{max} were calculated to be 4.0×10^{-6} M and 4.4×10^{-8} m moles/10^{8} cells/20 minutes, respectively. Guanosine inhibited the uptake of 2AG non-competitively. The inhibition constant (Ki) was 3×10^{-6} M (guanosine; 7.07×10^{-6} M).

Uptake of 2AG by Resistant and Insensitive Strains

The transport of 2AG-8-^{3}H into resistant strain KY 3591 (2AG^{r}) and insensitive strain K-12 is shown in Fig. 10. 2AG was taken up as it was by KY 3591 but guanine was not released upon uptake.

It is of interest that guanosine inhibited the uptake of 2AG by the resistant strain as it did in the sensitive strain KY 3591.

Discussion

It has been reported that the antibacterial activity of many nucleoside analogs including nucleoside antibiotics is reversed by the addition of nucleosides or bases.[4] The present study showed that 2AG acted on only two strains of *E. coli* KY 8323 and KY 3591 and its action was reversed by the addition of various nucleosides, especially by guanosine. Xanthosine and bases did not reverse inhibition by 2AG. Nucleotides such as AMP and GMP showed moderate or slight reversal but their action could be due to nucleosides produced during incubation.

When guanosine was added to the medium, the final growth level was proportional to the amount of guanosine added. In the presence of guanosine, 2AG was not transported into the cell and a preferential uptake of guanosine was observed. Guanosine inhibited the uptake of 2AG non-competitively indicating that 2AG was taken up through a mechanism different from that of guanosine.

It has been shown that purine nucleosides are transported into *E. coli* by purine nucleoside phosphorylase and purine phosphoribosyltransferases.[8] It appears that these enzymes are responsible for transporting guanosine into strain KY 3591, since guanine was released into the medium upon uptake of guanosine. However 2AG uptake did not result in guanine release. These results suggest the participation of guanosine kinase in the uptake of 2AG, which was recently proposed by JOCHIMSEN *et al.*[9]

DIVEKAR *et al.*[10] reported that a subline of mouse sarcoma 180 that is resistant to adenosine analogs showed a 20,000-fold reduction in adenosine kinase, indicating that adenosine kinase was also responsible for the incorporation of analogs. In contrast, 2AG resistant strain KY 3591 2AG^r and other resistant strains possessed the ability to take up 2AG and the uptake of 2AG was inhibited by guanosine. This observation indicates that the resistance to 2AG is not due to lack of transport into the cell. In a further study of the action mechanism of 2AG, it has become apparent that 2AG taken up in the cell is phosphorylated and then incorporated into RNA (in the following paper). Between sensitive and resistant strains, there is a difference in the ability to incorporate 2AG into RNA.

Acknowledgement

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