# The Effects of 6-Chloro-8-aza-9-cyclopentylpurine on Nucleic Acid and Protein Synthesis in *Escherichia coli*

## I. *In Vivo* Studies

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SUMMARY

6-Chloro-8-aza-9-cyclopentylpurine, a purine deoxyribonucleoside analogue, inhibited the synthesis of DNA, RNA, and protein, as well as the induction of β-galactosidase, in *Escherichia coli* B. Its effects were studied under conditions which allowed partial dissection of the inhibitions caused by this agent on the various macromolecular processes. *E. coli* B3, a thymidine-requiring mutant, and T2osr bacteriophage were employed for these purposes. The data indicate that the synthesis of DNA is more sensitive to 6-chloro-8-aza-9-cyclopentylpurine than is the formation of either RNA or protein. Blockade by this agent of the synthesis of RNA and protein appears to be independent of its effects on the processes involved in the formation of DNA. The induced synthesis of β-galactosidase is considerably more sensitive to the inhibitory effects of 6-chloro-8-aza-9-cyclopentylpurine than is the formation of total cellular protein. The results support the concept that there are multiple sites of blockade by the purine deoxyribonucleoside analogue.

### INTRODUCTION

The synthesis of 6-chloro-8-aza-9-cyclopentylpurine (Fig. 1), as well as the inhibition of the formation of steroid-induced enzyme synthesis (i.e., $\Delta^5$-3-ketosteroid isomerase) by this agent in *Pseudomonas testosteroni*, has been reported (1). The synthesis of nucleic acids and total protein in this organism was also shown to be affected by 689;[3] the magnitude, onset, and duration of inhibition of these metabolic processes differed, however, depending upon the concentration of 689 employed.

In the present study *Escherichia coli* B and B3, which can be propagated in a defined medium, as well as T2osr bacteriophage, were utilized to determine whether the inhibition of the formation of induced enzyme, nucleic acids, and total protein by 689 was the result of a single metabolic lesion, or whether several different bio-

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[3] The abbreviations used are: 689, 6-chloro-8-aza-9-cyclopentylpurine; DMSO, dimethyl sulfoxide.

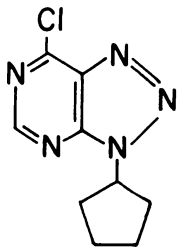

FIG. 1. *Structural formula of 6-chloro-8-aza-9-cyclopentylpurine*

chemical alterations were produced by this agent. The results show that blockade by this purine deoxyribonucleoside analogue of the synthesis of RNA and protein is distinct from its effects on the formation of DNA, indicating that 689 has inhibitory effects at multiple sites.

## MATERIALS AND METHODS

*Growth of bacteria. E. coli* B cells were maintained on C medium–agar plates and grown at 37° in C medium in which 0.2% glucose was used in place of maltose (2). *E. coli* B3 cells were maintained on enriched broth–agar plates supplemented with 40 μM thymidine. For experimental purposes, cells were employed in the early logarithmic phase of growth. Viability studies were performed by placing a dilution of the bacterial culture on C medium–agar plates and counting the resulting colonies 24 hr later. Turbidity measurements were performed using a Zeiss PMQ II spectrophotometer (660 mμ) or with a Klett-Summerson colorimeter (No. 66 filter).

*Compounds.* L-Leucine-1-$^{14}$C (34.2 μC/μmole) and uracil-2-$^{14}$C (30 μC/μmole) were purchased from New England Nuclear Corporation. Methyl-β-D-thiogalactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Mann Research Laboratories.

*Measurements of nucleic acid and protein synthesis.* The rate of incorporation of L-leucine-1-$^{14}$C into residual protein was used as a measure of the synthesis of total protein by methods described previously (1). The formation of RNA and DNA was ascertained by determining the rate of incorporation of uracil-2-$^{14}$C into alkali-labile material (0.5 N NaOH for 2 hr at 37°) and into alkali-resistant, hot acid-extractable material (5% trichloracetic acid for 30 min at 95°), respectively (3). The total content of DNA, RNA, and protein was measured as reported earlier (1). Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer using a scintillation mix of *p*-bis[2-(5-phenyloxazolyl)]benzene, 2,5-diphenyloxazole, toluene, and ethanol (1).

*Assay of β-galactosidase activity.* Bacteria were grown in a minimal medium containing salts and 1% (v/v) glycerol in place of glucose as the carbon source. The medium was supplemented with 1.5 μM thymidine in those experiments in which *E. coli* B3 was the test organism. β-Galactosidase was induced by the addition of 4 mM methyl-β-D-thiogalactopyranoside. At various intervals thereafter, 1-ml samples were removed, added to 0.1 ml of toluene, mixed well, and diluted with 1 ml of phosphate buffer (10 mM, pH 7.2). To 0.5 ml of the buffered enzymatic extract were added 2.5 ml of phosphate buffer containing *o*-nitrophenyl-β-D-galactopyranoside (1 mM) and $MgCl_2 \cdot 6H_2O$ (12 mM). Following incubation at 37° for 20 min, the mixture was heated at 95° for 1 min, and then 0.5 ml of a 1 M $K_2CO_3$ solution containing 10 mM EDTA was added. The intensity of the yellow color, representing free *o*-nitrophenol, was determined at 420 mμ.

*Bacteriophage experiments.* T2osr (osmotic shock-resistant, rapid lysis strain) bacteriophage were propagated in broth using *E. coli* B (4). Determination of the number of plaque-forming units and the number of infective centers was based upon procedures described by Adams (5). Adsorption buffer was prepared according to Hershey and Chase (6). T2 antiserum was prepared by the method of Sartorelli *et al.* (7). The degree of bacterial lysis was determined by measuring the decrease in absorbance of the bacterial suspension with a Klett-Summerson colorimeter, No. 66 filter.

Dimethyl sulfoxide was used to solubilize the 689; in each experiment, equivalent

amounts of this solvent were added to cultures which served as controls.

## RESULTS

*Effect of 689 on cell viability.* The number of *E. coli* B cells surviving exposure to 689 for various periods of time is shown in Fig. 2. Cells treated with a 0.5 mM concentration of the purine deoxyribonucleoside analogue for 1 hr were capable of forming colonies when the extracellular concentration of the compound was lowered by dilution. That no change occurred in the number of cells capable of forming colonies following exposure to 0.5 mM 689 for 60 min under the conditions employed indicated that the inhibition by 689 was immediate and suggested further that the drug was bacteriostatic (i.e., the cells were capable of recovery and subsequent cell division). Prolonged exposure to this agent at this concentration, however, resulted in apparently irreversible changes and subsequent cell death. At a higher level of drug (1 mM), approximately 80% of the cells were killed after exposure for only 1 hr.

*Effect of 689 on synthesis of nucleic acids and protein.* To gain information on the metabolic actions of this agent, the effect of 689 on the synthesis of DNA, RNA, and protein was measured isotopically by determining the rate of incorporation of uracil-2-$^{14}$C into DNA and RNA, and of leucine-1-$^{14}$C into residual protein (Fig. 3). DNA biosynthesis was rapidly and markedly inhibited at a concentration of 0.4 mM 689; the rates of formation of RNA and protein were affected to a lesser degree.

The findings obtained by isotopic measurements were corroborated by determining the effects of 1 mM 689 upon the total DNA, RNA, and protein content of cultures of *E. coli* (Table 1). These data indicate that the net accumulation of DNA was completely prevented by the analogue, whereas some formation of RNA and protein occurred in the presence of this agent. These observations were similar to those obtained in studies of the effects of 689 upon the synthesis of nucleic acids and protein in *P. testosteroni* (1).

To determine whether inhibition of the growth of *E. coli* by 689 could be reversed by deoxyribonucleosides, either deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine, or a mixture of these deoxyribonucleosides at a final concentration of 1 mM was added simultaneously with 1 mM

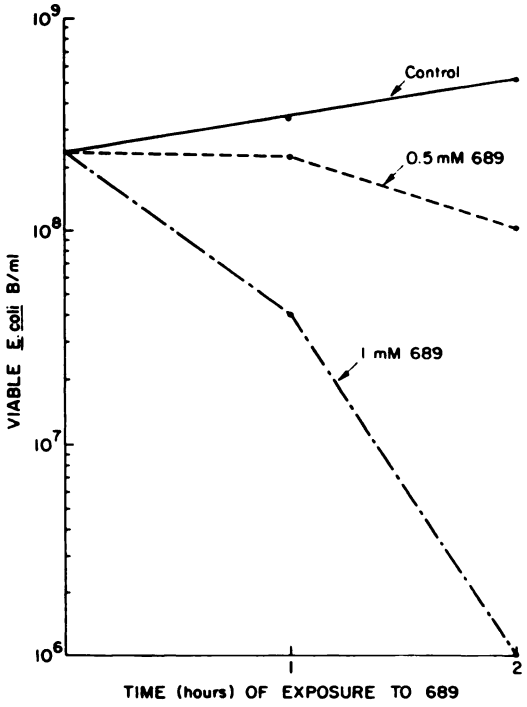


FIG. 2. *Effect of 689 on cell viability*

*E. coli* B cells were exposed to either 0.5 or 1 mM 689 for 1 or 2 hr, after which an aliquot of the suspension was diluted and placed on C medium–agar plates. Colony counts were performed 24 hr later.

TABLE 1

*Effect of 6-chloro-8-aza-9-cyclopentylpurine on total content of DNA, RNA, and protein in cultures of E. coli B*

6-Chloro-8-aza-9-cyclopentylpurine was added to a suspension of *E. coli* B cells in the logarithmic growth stage; 15 min later, samples were removed and analyzed for total DNA, RNA, and protein as described under MATERIALS AND METHODS.

| Treatment | DNA | RNA | Protein |
|---|---|---|---|
| | *μg/ml bacterial suspension* | | |
| Zero time | 5.0 | 22.8 | 40.5 |
| Control, 15 min | 6.6 | 28.8 | 52.0 |
| 1 mM 689, 15 min | 4.9 | 24.0 | 42.0 |

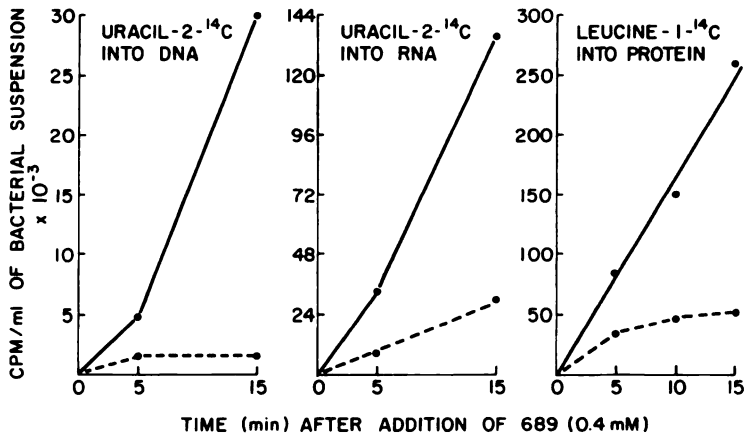


FIG. 3. *Effect of 689 on DNA, RNA, and protein synthesis in E. coli B*

Uracil-2-$^{14}$C (30 $\mu$C/$\mu$mole) or leucine-1-$^{14}$C (34.2 $\mu$C/$\mu$mole) was added simultaneously with 0.4 mM 689. After the designated intervals of time, aliquots were removed for analysis as described in MATERIALS AND METHODS. ●——●, DMSO control; ●- - -●, 689 (0.4 mM).

689. Two hours later, the optical density of the cultures at 660 m$\mu$ was determined. The inhibition of cell growth by 689 was not reversed by any of the four deoxyribonucleosides used alone or in combination (0.25 mM concentration of each deoxyribonucleoside). The deoxyribonucleosides themselves had no effect on growth under the conditions employed.

The action of 689 on the induced synthesis of $\beta$-galactosidase in *E. coli* was similar to its effect on the induction of $\Delta^5$-3-ketosteroid isomerase in *P. testosteroni* (1). Induction of $\beta$-galactosidase in *E. coli* B was completely inhibited 3 min after the addition of 0.5 mM 689; in contrast, the synthesis of total protein, measured isotopically, was inhibited approximately 60% after exposure to 689 for 10 min.

*Studies on mechanism of inhibition of nucleic acid and protein synthesis.* In an attempt to determine whether 689 was capable of producing inhibitory effects at multiple sites or whether the effects observed were the result of metabolic changes in response to a single drug-induced biochemical alteration, various techniques were employed in an effort to dissect individual parameters. *E. coli* B3, a thymidine-requiring mutant of *E. coli* B, was used for the purpose of determining whether 689 would produce lesions in the biosynthetic pathways involved in the formation of RNA and protein in the absence of the net synthesis of DNA. As indicated in Fig. 4, 30 min after the removal of thymidine from the medium, the biosynthesis of DNA in *E. coli* B3 cells stopped, while the formation of RNA, protein, and $\beta$-galactosidase in response to an inducer continued unabated. The addition of 0.5 mM 689 to the culture at this time resulted in the immediate and complete termination of the formation of both RNA and induced enzyme; in contrast, the synthesis of protein continued at the control rate for 15 min before slight inhibition ensued. These data indicate that the blockade by 689 of the synthesis of RNA and $\beta$-galactosidase can occur in the absence of DNA biosynthesis, and is not due to a decrease in the total amount of DNA.

It was conceivable that 689 interfered with the ability of preformed DNA to function as a template. The finding that prior incubation of DNA with 689 did not result in a decrease in the ability of DNA to function as a template for RNA polymerase appeared to eliminate this possibility (8); however, such a conclusion assumes that activation of the drug to a reactive form does not occur *in vivo*. Further evidence to support the contention

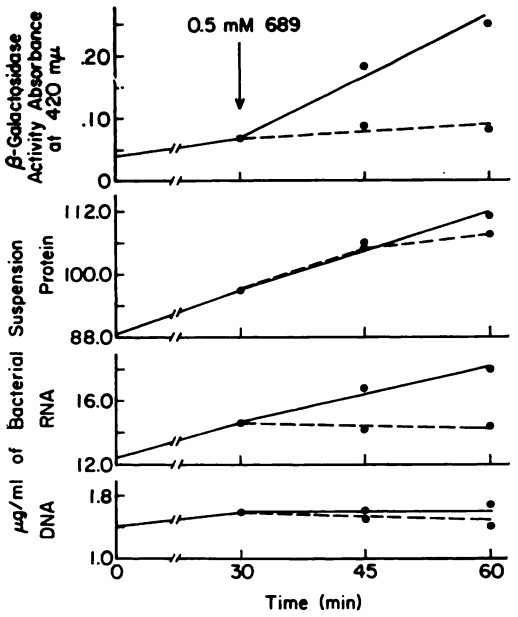


FIG. 4. *Effect of 689 on the total content of DNA, RNA, protein, and induced β-galactosidase in E. coli B8.*

Cells were grown in a minimal salts–glycerol medium supplemented with 1.5 μM thymidine. During early logarithmic growth, cells were harvested, washed, and resuspended in thymidine-free medium. Methyl-β-D-thiogalactopyranoside (4 mM), an inducer of β-galactosidase, was added, and 30 min later the culture was divided; either 0.5 mM 689 or the solvent DMSO was added, and aliquots were withdrawn 15 and 30 min later. These samples were analyzed for total DNA, RNA, protein, and β-galactosidase activity as outlined in MATERIALS AND METHODS. ●——●, DMSO control; ●- - -●, 689 (0.5 mM).

that the decrease in the rate of formation of RNA and protein caused by the purine deoxyribonucleoside analogue was independent of the effects of this agent on the biosynthesis of DNA was obtained by infecting *E. coli* cells with T2osr bacteriophage. The results shown in Fig. 5 indicate that 689 is capable of inhibiting the decrease in absorbance caused by T2osr bacteriophage-induced lysis of *E. coli* B. To investigate the mechanism by which the purine deoxyribonucleoside analogue interfered with phage-induced lysis of *E. coli*, the effect of this agent on lysozyme activity was ascertained indirectly by addition of inhibitor at various intervals of time after phage infection. Minimal inhibition of cell lysis was obtained when 0.3 mM 689 was added approximately 15–20 min after the addition of the phage particles to the cell suspension. At this time, under normal conditions, infective phage particles are detectable, although an additional interval of about 5–10 min is required before maximum lysozyme activity is attained (9). These data indicated that 689 had little or no effect on lysozyme activity, and suggested that the inhibitor was acting on processes involved in the formation of the phage particle.

Incubation of the phage particles with 0.4 mM 689 prior to the addition of the phage to the bacterial culture did not result in inhibition of cell lysis, suggesting that this agent did not interfere with phage-induced lysis of *E. coli* by alteration of either the phage coat protein (an effect of 689 on phage coat protein might prevent attachment of the phage to the bacterium) or phage DNA (assuming that the phage particles are permeable to the drug). The data in Table 2 show that prior treatment of *E. coli* B with 0.4 mM 689 for either 5

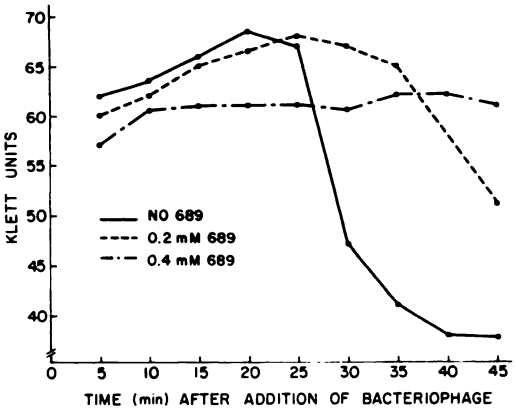


FIG. 5. *Effect of 689 on T2osr bacteriophage-induced lysis of E. coli B*

To a suspension of *E. coli* B in the early logarithmic phase of growth was added T2osr bacteriophage at a multiplicity of infection of 2, together with either 0.2 or 0.4 mM 689. The absorbance of the suspension was then measured at intervals with a Klett-Summerson colorimeter (No. 66 filter).

TABLE 2

*Ability of T2osr bacteriophage to infect and replicate in E. coli B cells treated with 6-chloro-8-aza-9-cyclopentylpurine*

After a 5- or 15-min exposure of *E. coli* B cells ($4 \times 10^8$ cells) to 689 (0.4 mM), the cells were harvested, washed, resuspended in adsorption buffer, and infected with T2osr phage at a multiplicity of infection of about 0.5. After 10 min had been allowed for adsorption of the phage and injection of their DNA, T2 antiserum was added to remove any unattached phage particles. After 5 min of incubation at 37°, the cells were harvested and washed, and the number of infective centers was determined. Lysis of the infected bacteria was monitored, and the number of plaque-forming units (pfu) after lysis was also determined (see MATERIALS AND METHODS).

| Expt. | Duration of 689 treatment of *E. coli* B | Total infective centers | | Total pfu after lysis | | pfu/infective center | | Inhibition by 689 |
|---|---|---|---|---|---|---|---|---|
| | | Control | 689 | Control | 689 | Control | 689 | |
| | *min* | $\times 10^{-9}$ | | $\times 10^{-10}$ | | | | % |
| A | 5 | 1.98 | 1.94 | 12.1 | 5.00 | 61.3 | 25.8 | 57.8 |
| B | 5 | 1.92 | 2.59 | 5.80 | 4.50 | 30.2 | 17.4 | 42.3 |
| | 15 | 2.64 | 2.00 | 10.1 | 1.09 | 38.3 | 5.45 | 85.8 |
| C | 5 | 1.50 | 1.22 | 5.85 | 2.25 | 39.0 | 18.5 | 52.5 |
| | 15 | 1.11 | 1.17 | 8.20 | 1.10 | 74.0 | 9.4 | 87.3 |

or 15 min also did not interfere with either the attachment of phage particles to the bacteria or injection of phage DNA into the host cells. Thus, an equal number of infective centers were produced in both the 689-treated bacterial suspension and the bacterial suspension treated only with DMSO. This indicated that in both cultures an equal number of bacteria were infected with phage and that at least 1 plaque-forming unit was formed per phage-infected bacterium. The total number of plaque-forming units present in the bacterial suspension after cell lysis was greatly reduced, however, in those cultures initially treated with the inhibitor. Approximately 50% and 86% inhibition of the number of plaque-forming units formed per infective center occurred in those bacteria treated with 689 for 5 and 15 min, respectively. Furthermore, the degree of inhibition of the synthesis of DNA, RNA, and protein caused by the treatment of *E. coli* with 689 was not influenced by the addition of phage, as determined with radioactive precursors of the nucleic acids and proteins. This indicated that the addition of phage particle DNA to the bacteria did not reverse the inhibition of RNA and protein synthesis which remained after the cells had been washed to remove excess 689. These data support the concept that the inhibition by this agent of RNA and protein synthesis in the *E. coli* host cell is not due to any direct alteration by the drug of bacterial DNA. Employing $^{14}$C-labeled 689 (10), it was determined that only 1% of the total radioactivity added to the medium as $^{14}$C-689 was associated with the cells after washing; furthermore, this radioactivity could not be removed by treatment of the bacterial suspension with cold perchloric acid. Although this level of drug may represent an intracellular concentration of 689 of about 1 mM, based on the approximate cell volume, the compound appeared to be firmly bound and, therefore, presumably was not readily available to interact with the phage DNA injected subsequent to the washing procedure.

## DISCUSSION

6-Chloro-8-aza-9-cyclopentylpurine markedly inhibits the biosynthesis of DNA, RNA, and protein; the most sensitive of these metabolic processes to the inhibitory activity of this agent is the formation of

DNA. Studies on the site of the metabolic lesion induced by 689 on the DNA-biosynthetic pathways of *E. coli* have shown that the purine deoxyribonucleoside analogue interferes with the formation of thymidylate (8). Although the precise mechanism involved in the blockade of thymidylate synthesis by 689 is unknown, it would appear that the magnitude of this effect is sufficient to account for the inhibition of DNA biosynthesis *in vivo*. The absence of inhibition *in vitro* of DNA polymerase and thymidylate synthetase by 689, as well as the observation that DNA treated with this agent functions effectively as a template in both the DNA and RNA polymerase reactions (8), supports the concept that a drug-imposed decrease in the supply of thymine nucleotides is responsible in part for the inhibitory action of this agent on the biosynthesis of DNA.

The finding that the formation of RNA in *E. coli* B3 cells deprived of an external source of thymidine, and therefore incapable of synthesizing DNA, was sensitive to the inhibitory action of 689 indicated that blockade of the formation of RNA by this agent did not require the active net synthesis of DNA. The results obtained with the bacteriophage experiments, in which the addition of untreated phage (which in essence is equivalent to insertion of DNA into microbial cells) to 689-treated *E. coli* was not capable of reversing the inhibitory effects of 689, give further support to the concept that the effects of the purine deoxyribonucleoside analogue on RNA were not due to its action on bacterial DNA. That the purine deoxyribonucleoside analogue is capable of producing separate metabolic alterations of both the RNA- and protein-synthesizing systems was corroborated by the finding that 689 inhibits RNA polymerase activity and polypeptide synthesis in cell-free extracts of *E. coli* (8).

The sensitivity of β-galactosidase to 689 was not due to direct inhibition of this enzyme by this agent, since incubation of a cell-free extract of *E. coli* with 2 mM 689 for 6 min following induction of β-galactosidase with methyl-β-D-thiogalactopyranoside did not result in inhibition of enzymatic activity. These results are in agreement with the pronounced sensitivity of the steroid-induced formation of $\Delta^5$-3-ketosteroid isomerase to 689 in *P. testosteroni*. Following exposure to drug, the latter organism recovered the capacity to synthesize total protein at a rate greater than that of the induced synthesis of isomerase (1).

The molecular configuration of 689 is rather critical for the production of the specific effects observed *in vivo*. Minor modifications in either position 6 or 8 of the purine ring result in a marked loss of activity (1, 10). The presence of a chlorine atom in position 6 of the purine nucleus of 689 confers upon this agent the capacity to alkylate. However, since the structure of 689 resembles that of a purine deoxyribonucleoside, it is envisioned that this compound is capable of fitting the active centers of a variety of enzymes, and therefore any alkylation of these enzymes which ensues following binding of 689 may be considered to be site-directed. Evidence for the formation of a covalent bond between 689 and cellular components is provided by the finding that a considerable quantity of radioactivity from $^{14}$C-labeled 689 remains associated with the residue of *E. coli* cells not solubilized by treatment with 0.4 N perchloric acid. Mitomycin C, also shown to be capable of alkylation, has been found to produce effects in *E. coli* similar to those produced by 689 (11).

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