useful agents for the study of the consequences of protein synthesis errors in vivo and for the investigation of the genetics of ribosomes in a lower eukaryote.

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Aminoglycoside Antibiotics and Eukaryotic Protein Synthesis: Stimulation of Errors in the Translation of Natural Messengers in Extracts of Cultured Human Cells†

James M. Wilhelm,*,‡ John J. Jessop, and Susan E. Pettitt

ABSTRACT: Aminoglycoside antibiotics were tested for the capacity to stimulate misreading in a cell-free protein synthesis system derived from human cells (line KB). The stimulation of leucine incorporation with a poly(U) template in preincubated extracts was employed as one test of misreading. Some of the 2-deoxystreptamine-containing antibiotics stimulated misreading; high misreading activity was correlated with the presence of a paromamine (or 3-deoxyparomamine) moiety in the molecule. Thus, paromomycin and lividomycin B stimulated considerably more misreading than neomycin. Members of the streptomycin family had no activity in this system. The effects of paromomycin were further examined in a system from cells infected with type 5 adenovirus. In the system, protein synthesis is dependent on endogenous messengers and the translation product, which is composed of adenovirus polypeptides, can assemble into multimeric protein complexes, characteristic of viral substructures (Wilhelm, J. M., & Ginsberg, H. S. (1972) *J. Virol. 9,* 973). The presence of paromomycin, during protein synthesis, markedly reduced the capacity of the polypeptides made to participate in the assembly reaction. Furthermore, evidence was obtained which suggests that paromomycin may cause the insertion of methionine into a particular protein which normally contains little or none of that amino acid. The results suggest that an aminoglycoside antibiotic can stimulate misreading of natural messengers in a human cell system.

Many of the aminoglycoside antibiotics promote mistranslation with bacterial ribosomes; in contrast, the specific antibiotic, streptomycin, does not stimulate protein synthesis errors with cytoplasmic ribosomes of eukaryotic cells (Weinstein et al., 1966; Friedman et al., 1968; Kurtz, 1974). Our laboratory has undertaken a systematic study of aminoglycosides and protein synthesis in eukaryotic systems. Evidence has been presented (Wilhelm et al., 1978) on the stimulation of mistranslation by a specific subset of aminoglycosides, namely, paromomycin and related molecules containing the fragment, paromamine. Those studies demonstrated errors in

† From the Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received July 22, 1977.* Supported by National Institutes of Health Grant AI-11427. A preliminary account of portions of this work has been communicated (Wilhelm & Jessop, 1975).

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the translation of poly(U)[1] with a wheat embryo system. In the present communication we report that paromomycin stimulates errors during translation of natural messenger RNA in a cell-free system from human cells.

Materials and Methods

Materials. Biochemicals were products of Sigma. Radiolabeled compounds (specific activity in Ci/mmol) were obtained as follows: [^{3}H]phenylalanine (1.1), ICN; [^{14}C]leucine (0.328), [^{14}C]arginine (0.310), [^{35}S]methionine (380), NEN. Sources of antibiotics were noted previously (Wilhelm et al., 1978).

Cell Cultures and Virus. Methods for growth of the human cell line KB and propagation of type 5 adenovirus have been described (Bello & Ginsberg, 1969). Cells were collected from suspension cultures and washed twice with cold 10 mM Tris-HCl, pH 7.6, containing 150 mM NaCl. When infected cells were desired, the cultures were infected with 150 plaque-forming units of type 5 adenovirus per cell 18 to 20 h before collection.

Preparation of Cytoplasmic Extracts. Washed cells (3–5 $\times 10^8$) were suspended in cold homogenizing buffer (10 mM Tris-HCl, pH 7.6, 1.5 mM magnesium acetate, 10 mM KCl, 6 mM mercaptoethanol; 1 mL for 8×10^7 cells), allowed to swell for 10 min, and disrupted in a Dounce homogenizer. Nuclei and unbroken cells were removed by two cycles of centrifugation at 1100*g* for 10 min. The supernatant was adjusted by addition of a 10× solution to the following buffer and ion concentrations: 20 mM Tris-HCl, pH 7.6, 5 mM magnesium acetate, 80 mM KCl. The solution was applied to a column (15 × 25 cm) of Sephadex G-25, and the column was developed with the above buffer containing 6 mM mercaptoethanol. The turbid material eluting in the void volume was collected, adjusted to 40 A_{260} units/mL, and frozen in liquid nitrogen.

The preparation of preincubated extracts proceeded as above through centrifugation at 1100*g*. The extract was then incubated at 30 °C for 40 min with the following components: 20 mM Tris-HCl (pH 7.6), 3.5 mM magnesium acetate, 120 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 6.6 mM creatine phosphate (potassium salt), 100 μg/mL phosphocreatine kinase, and 20 μM each of 20 amino acids. After incubation, the solution was centrifuged at 30 000*g* for 10 min, and the supernatant was subjected to gel filtration.

Amino Acid Incorporation with Cytoplasmic Extracts. Reaction mixtures of 25 to 250 μL contained the following: 0.6 volume of extract, 50 mM Tris-HCl (pH 7.6), 3 mM magnesium acetate, 148 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 6.6 mM creatine phosphate, 100 μg/mL phosphocreatine kinase, 2.5 μCi/mL (7.8 μM) [^{14}C]leucine, or 2.5 μCi/mL (8.0 μM) [^{14}C]arginine, or 100 μCi/mL (2.0 μM) [^{35}S]methionine, and 100 μM each of the appropriate 19 unlabeled amino acids. The final concentration of [^{35}S]methionine was obtained by addition of unlabeled methionine. After incubation for 40 min at 30 °C, the solution (or a portion thereof) was diluted into 2–3 mL of 5% Cl_3CCOOH and placed in boiling water for 10 min, and the precipitate was filtered onto a nitrocellulose filter. The filters were washed with Cl_3CCOOH and 80% ethanol. Incorporated radioactivity was determined by scintillation spectrometers.

For experiments with poly(U), reaction mixtures of 25 μL were as described above with the following modifications: 0.6 volume of a preincubated extract; 8 mM magnesium acetate; 50 μg/mL poly(U); 12.5 μCi/mL (1.3 μM) [^{3}H]phenylalanine, or 2.5 μCi/mL (7.8 μM) [^{14}C]leucine, and 100 μM of the 19 appropriate unlabeled amino acids.

Gel Electrophoresis of Polypeptides from in Vitro Synthesis Reactions. After incubation for protein synthesis, reaction mixtures were made 0.1 M in NaOH and held at 37 °C for 1 h. A 10–20-fold excess volume of cold 5% Cl_3CCOOH was added and the precipitates were collected by centrifugation. Precipitates were washed twice in Cl_3CCOOH, twice in acetone, dried briefly, and dissolved in a denaturing solution (0.05 M Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% $NaDodSO_4$, 10% glycerol). Samples were solubilized by heating in boiling water for 2 min. Electrophoresis on 10% polyacrylamide gels was performed either in glass tubes (6 mm i.d.) or with a slab gel apparatus. The $NaDodSO_4$–Tris system described by Weber & Osborn (1975) was used. Gels from tubes were sliced into 2-mm fractions and material in the fractions was solubilized into Protosol (New England Nuclear) and assayed for radioactivity according to the manufacturer's directions. Slab gels were dried and autoradiograms were prepared by exposure to x-ray film (Kodak SB54).

Sucrose Density Sedimentation of Proteins. Samples (100–200 μL) of in vitro incubation mixtures or reference markers were layered on 5 mL, 5–20% linear sucrose gradients in 10 mM phosphate buffer (pH 7.6), 150 mM NaCl, 1 mM EDTA. Gradients were centrifuged for 19.5 h at 20 °C in a Beckman SW 50.1 rotor at 26 000 rev/min. Fractions of approximately 125 μL were collected directly onto filter paper disks which were washed with hot and cold 5% Cl_3CCOOH, 80% ethanol, dried, and assayed for radioactivity. Material pelleted into the bottom of the tube was solubilized into 0.5% $NaDodSO_4$ and applied to a disk. Sedimentation coefficients were estimated from the positions of type 5 adenovirus fiber and hexon capsomers in parallel centrifugations of extracts of radiolabeled infected cells. $s_{20,w}$ for fiber and hexon are 6.1 and 12.1 (Levine & Ginsberg, 1967).

Results

Misreading of Poly(U). With cytoplasmic extracts of human cells (line KB), which had been preincubated to reduce endogenous template activity, the incorporation of phenylalanine into polypeptide was stimulated some 60-fold by poly(U). In a typical experiment with a 25-μL system, incorporation was 600 cpm without poly(U) and 37 000 cpm with the template. Most aminoglycoside antibiotics did not inhibit phenylalanine incorporation; only neomycin was inhibitory at high concentration (70% inhibition at 280 μM).

Poly(U) does not contain codons for leucine; the template stimulated leucine incorporation only very slightly (without poly(U), 400 cpm; with poly(U), 600 cpm). Leucine incorporation can be used as an assay of misreading, and the misreading potential of several aminoglycosides was tested (data not shown). Paromomycin (Pm)[1] and lividomycin B stimulated leucine incorporation sevenfold at 140 μM, but neomycin stimulated less than twofold. Kanamycin C (300 μM) elevated leucine errors threefold, whereas kanamycins A and B were much less active (about 20% stimulation). Streptomycin, and the related drug, bluensomycin, did not stimulate leucine errors at all.

Kanamycin C, lividomycin B, and Pm are structurally related; each contains a paromamine moiety (3-deoxyparomamine in lividomycin B). This moiety is the common structural feature of the aminoglycosides that elicit strong misreading of poly(U) in another eukaryotic system, the cytoplasmic extract of wheat embryos (Wilhelm et al., 1978).

Misreading of Natural Messengers. The remaining ex-

[1] Abbreviations used: poly(U), poly(uridylic acid); Pm, paromomycin; $NaDodSO_4$, sodium dodecyl sulfate.

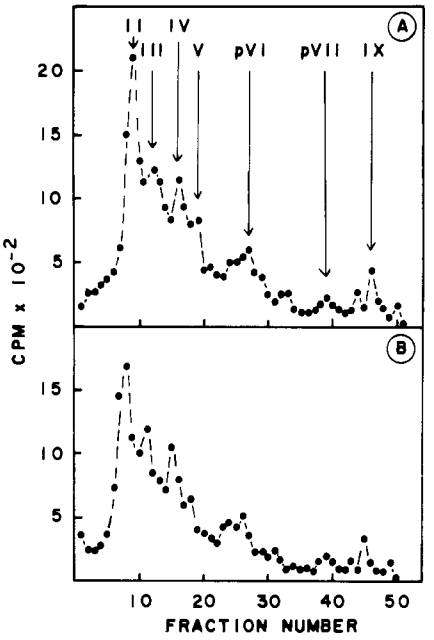

FIGURE 1: Gel electrophoretic analysis of the polypeptides synthesized in cell-free systems from adenovirus-infected cells; comparison of the standard system with synthesis in the presence of paromomycin. (Part A) Standard synthesis system (100 μL reaction volume); radiolabel was [^{14}C]leucine. Total radioactivity applied to gel: 29 600 cpm (equivalent to 0.6 of translation product). (Part B) Synthesis system (100 μL) with Pm (280 μM); total radioactivity applied to gel, 22 000 cpm (0.6 of product). Further experimental details may be found in Materials and Methods. The Roman numeral designations of adenovirus polypeptides generally follow conventions originated by Maizel et al. (1968).

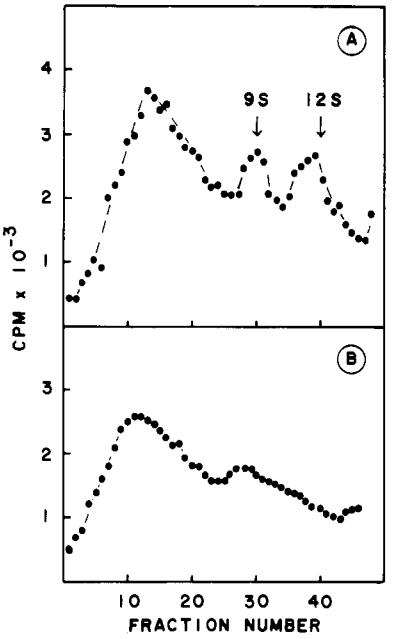

FIGURE 2: Sedimentation analysis of the polypeptides synthesized in cell-free systems from adenovirus-infected cells; comparison of the standard system with synthesis in the presence of paromomycin. (Part A) Standard synthesis system (250 μL reaction volume); radiolabel was [^{14}C]leucine. Total radioactivity applied to gradient: 124 000 cpm (200 μL of incubation). (Part B) Synthesis system (250 μL) with Pm (280 μM); total radioactivity applied to gradient, 82 000 cpm (200 μL). Further experimental details may be found in Materials and Methods.

periments presented in this communication employed cell-free systems that were not preincubated and to which no endogenous template was added. Polypeptide synthesis in these systems depended on endogenous mRNA. When the cells are infected with adenovirus, the cell-free system synthesizes adenovirus polypeptides (Caffier et al., 1971; Caffier & Green, 1971; Wilhelm & Ginsberg, 1972). The translation product of an infected-cell system, analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, may be seen in Figure 1, part A.

In the intact cell, adenovirus polypeptides are ultimately assembled into viral substructures, or capsomers (Velicer & Ginsberg, 1970), and into mature virus particles. Detailed discussions of the location of polypeptides in the virus have been presented by others (e.g., Everitt et al., 1975). Relevant to the present work is a demonstration that polypeptides made in the cell-free system are capable of partial assembly into multimeric structures, which have sedimentation properties of adenovirus capsomers (Wilhelm & Ginsberg, 1972). In the experiment of Figure 2, part A, the product of an infected-cell system was centrifuged on a sucrose density gradient. In such analyses, about 10–15% of the incorporated label was not released from ribosomes. Of the soluble product, one-half was found as species of 9 and 12 S, and the rest as a peak at 3 S. Previous experiments with specific antisera demonstrated that the 9S peak contained adenovirus penton base capsomers and the 12S peak contained hexon capsomers (Wilhelm & Ginsberg, 1972). In more recent experiments (Wilhelm, unpublished), the composition of these peaks was examined by gel electrophoresis. The 12S material contained only polypeptides II and pVI. II is the major component of the hexon and VI (the cleavage produce of pVI) is associated closely with the hexon in the assembled virus (Everitt et al., 1973, 1975). About one-half of the 9S material was polypeptide III, the subunit of the penton base; the remainder was polypeptide II. Since a monomeric form of II (molecular weight approximately 105 000) would not be expected at this sedimentation value, the 9S form may be an assembly intermediate. Polypeptide II was also detected in 9S form after a brief amino acid label with intact infected cells (Wilhelm, unpublished).

The major polypeptide IV was always found in the 3S region of the gradient, apparently as free chains. This polypeptide, however, is the subunit of the adenovirus fiber, which in the assembled form (6 S) is probably a trimer (Dorsett & Ginsberg, 1975) in which two of the chains are glycoproteins (Ishibashi and Maizel, 1974). Therefore, a complex pathway involving membranes and possibly the cell nucleus may be involved in fiber assembly; such structures are not present in a cytoplasmic extract.

It is important to realize that the fraction of the ribosome-free translation product that participates in the assembly reaction is independent of the actual level of cell-free synthesis. This may be understood if one considers that cell-free synthesis provides only *tracer* quantities of labeled polypeptides, which mix into, and assemble from, preexisting pools of unlabeled polypeptides.

The assembly reaction of viral polypeptides might be disturbed if those polypeptides contained translation errors. Therefore, the action of Pm on the infected-cell system was studied. Overall polypeptide synthesis, measured as leucine incorporation, was slightly inhibited by Pm (20% inhibition with 280 μM Pm). The patterns of polypeptides synthesized with and without Pm were analyzed by gel electrophoresis (Figure 1). Without Pm, the relative amounts of the total labeled polypeptides which migrated as II, III, and pVI were 26, 14, and 5%, respectively. With 280 μM Pm, these values were 25, 14, and 5. Therefore, Pm did not selectively reduce the synthesis of those polypeptides which are the major partici-

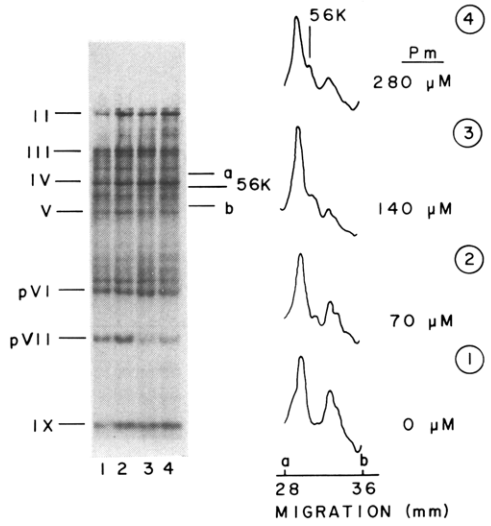

FIGURE 3: Autoradiograms of gel electrophoretic analyses of polypeptides synthesized in cell-free extracts of adenovirus-infected cells; the effect of increasing concentrations of paromomycin. Incubation volumes were 50 μL and the radiolabel was [^{35}S]methionine. Pm was present as indicated. For each panel, 300 000 cpm (equivalent to 0.1 of the translation product) was applied to the gel slot. The dried gels were exposed to film for 24 h. Roman numerals designate adenovirus structural proteins (or precursors). The appearance of the band designated 56K (56 000) is discussed in the text. The region a to b appears as the densitometer trace. Panels 1, 2, 3, 4: Pm at 0, 70, 140, and 280 μM, respectively. Further experimental details may be found in Materials and Methods.

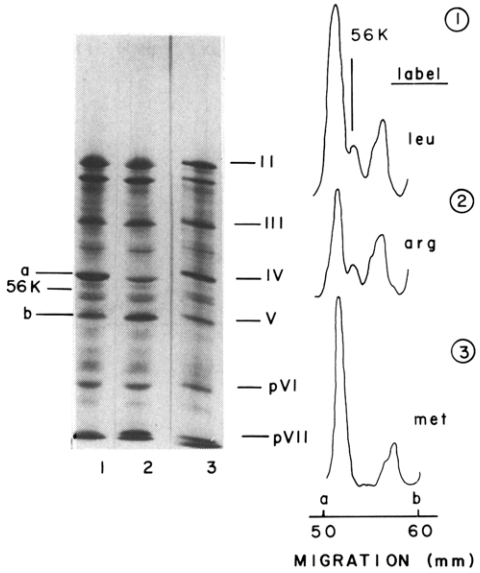

FIGURE 4: Autoradiograms of gel electrophoretic analyses of polypeptides synthesized in cell-free extracts of adenovirus-infected cells; comparison of polypeptide patterns labeled with leucine, arginine, and methionine. Incubation volumes were 150 μL. In the case of the system labeled from methionine, the solubilized polypeptides were diluted 1 to 10 with a solubilized, *unlabeled,* extract so that comparable quantities of total protein would be applied to gels. For these analyses, polyacrylamide gels of 7.5%, rather than the usual 10%, were employed. The dried gels were exposed to film for 2 weeks. Roman numerals designate adenovirus structural proteins (or precursors). The band designated 56K (56 000) is discussed in the text. The region a to b appears as the densitometer trace. (Panel 1) Labeled from [^{14}C]leucine; 34 000 cpm applied to gel. (Panel 2) Labeled from [^{14}C]arginine; 28 000 cpm applied to gel. (Panel 3) Labeled from [^{35}S]methionine; 41 000 cpm applied to gel. Further experimental details may be found in Materials and Methods.

pants in the assembly reaction. However, the formation of the protein complexes was markedly altered by Pm (Figure 2). In the control experiment, 47% of the ribosome-free polypeptide sedimented as complexes (>7 S). From the reaction with Pm, only 33% was found as complexes; no distinct 12S peak was observed and the 9S peak was ill defined. The alteration in assembly was only observed if Pm was present *during* protein synthesis.

The polypeptides synthesized in extracts were analyzed by electrophoresis in gel slabs so that better resolution of the pattern could be obtained by autoradiography. For these experiments, the polypeptides were labeled with [^{35}S]methionine. The autoradiograms of Figure 3 show the patterns observed with and without Pm. Polypeptides were made in Pm which migrate at the positions characteristic of the viral species; there was no relative change in the major bands. However, there was one easily discernible alteration in the pattern; in the presence of Pm, a new polypeptide band with an approximate molecular weight of 56 000 appeared (marked 56K). The new polypeptide band may be seen in densitometer tracings of the relevant portions of autoradiograms.

We have tentatively postulated that the 56 000 (56K) polypeptide is always made by the system but that it normally contains little or no methionine. This hypothesis was suggested by an observation made in passing by Lindberg & Sundquist (1974). These workers identified four KB cell polypeptides which were components of messenger ribonucleoprotein complexes and which were also synthesized in adenovirus-infected cells. These polypeptides may be cellular species whose synthesis is retained during viral infection. One polypeptide, approximate molecular weight 56 000, was barely discernible when the cells were labeled from methionine alone.

An experiment was designed to detect the 56 000 polypeptide in the normal translation product of the infected-cell system. Three radiolabels were used, methionine, arginine, and leucine, and the polypeptides were analyzed on more porous polyacrylamide gels for better resolution. The results may be seen as the autoradiograms and tracings of Figure 4. The 56 000 species was clearly observed in the pattern labeled from leucine or arginine but could not be detected in the pattern labeled from methionine.

Discussion

Experiments reported here show that certain aminoglycoside antibiotics stimulate errors in the translation of poly(U) by a human cell-free system. Translation errors were detected as the stimulation of leucine incorporation; leucine codons, CUN or UUpurine, are not contained in poly(U). Streptomycin does not induce misreading activity in this system; our results are thus fully consistent with conclusions from earlier studies of streptomycin and eukaryotic protein synthesis (Weinstein et al., 1966; Friedman et al., 1968; Kurtz, 1974). However, we find that other drugs are active and that high activity is specifically associated with those molecules, like paromomycin, containing a paromamine moiety.

Apart from studies with a poly(U) template, we have obtained evidence for stimulation of errors in the reading of natural messengers. The endogenous translation product of a cytoplasmic extract of cells infected with adenovirus is principally composed of viral-specific polypeptides. These polypeptides are capable of assembly in a cell-free system into complexes which are, or are at least related to, substructures of mature virus particles (Figures 1 and 2; also Wilhelm & Ginsberg, 1972). One might expect the assembly of these polypeptides to be dependent on the proper amino acid sequence of the chains, and, indeed, if amino acid analogues are supplied to intact cells or to cell-free systems, assembly is reduced (Wilhelm, unpublished observations). The presence of Pm during cell-free synthesis markedly reduced the potential for

assembly (Figure 2); the results suggest that the drug can provoke errors during the translation of messengers for the viral polypeptides.

Finally, we have made some observations concerning the translation of a specific polypeptide (approximate molecular weight, 56 000; designated 56K). The nature of this polypeptide is discussed above in Results. We emphasize here that the 56 000 species appears to contain little or no methionine (Figure 4). However, a methionine-labeled 56 000 species appeared in the product of cell-free synthesis upon the inclusion of Pm (Figure 3). In another set of experiments (Wilhelm, in preparation) messenger RNA was extracted from adenovirus-infected cells and translated in a wheat germ cell-free system. A methionine-labeled polypeptide with a molecular weight of 56 000 appeared in the presence of certain specific aminoglycoside antibiotics: those containing paromamine (or 3-deoxyparomamine), which have been shown to be highly active in stimulating mistranslation of poly(U). We suggest that the appearance of methionine in the 56 000 species could arise by the misreading of UUG as AUG. This suggestion is reasonable, inasmuch as Pm stimulates isoleucine as well as leucine errors in the translation of poly(U); these most likely arise by misreading UUU as AUU (Wilhelm et al., 1978).

The perturbations that we have observed in the synthesis and assembly of specific proteins in a cell-free system should in principle be detectable in intact cells, if the drug can be introduced into the cells. Experiments are now in progress to this end.

Note Added in Proof

Recent experiments have demonstrated that Pm causes phenotypic suppression of several yeast nonsense mutants (E. Palmer and J. M. Wilhelm, manuscript in preparation). Therefore, we would suggest, as an alternative possibility, that the 56K species arises by read through of a termination codon with Pm.

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