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Association of 16S and 23S ribosomal RNAs to form a bimolecular complex

(prokaryotic protein synthesis/ribosomal RNA function/ribosomal RNA–RNA interaction)

D. P. BURMA, B. NAG, AND D. S. TEWARI

Molecular Biology Unit, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India

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ABSTRACT **Association of the 30S and 50S subunits to generate the 70S ribosomes of *Escherichia coli* has long been known but the mechanism of this interaction remains obscure. Light-scattering studies indicate that naked 16S and 23S RNAs can also associate under conditions similar to those required for the assembly of ribosomes from the constituent RNAs and proteins. The RNA–RNA association also takes place in the presence of ethanol, which promotes folding of 16S and 23S RNAs into specific compact structures with the morphological features of 30S and 50S ribosomes, respectively. Equimolar amounts of the two RNAs are involved in the association. The formation of a stoichiometric complex was shown by light scattering, sucrose density gradient centrifugation, and composite polyacrylamide/agarose gel electrophoresis. The presence of the two species of RNA in the complex was also shown by gel electrophoresis. The association of naked 16S and 23S RNAs suggests that RNA–RNA interaction may play an important role in the association of 30S and 50S subunits.**

It has been known for 25 years that the *Escherichia coli* 70S ribosome is composed of two subunits, the 50S subunit and the 30S subunit (1), that dissociate at low Mg^{2+} concentrations. During protein synthesis, the two subunits associate at the beginning of chain elongation and then dissociate at chain termination (2). The mechanism of this association and dissociation is unknown. Traub and Nomura (3) have shown that the 30S subunit can be reconstituted from 16S RNA and the 30S ribosomal proteins in the presence of high concentrations of salt and Mg^{2+}. These conditions are referred to as reconstitution conditions. Assembly of the 50S subunit, on the other hand, has required a more elaborate two-step procedure (4–6).

In this laboratory, RNase I has been used to study the structure of *E. coli* ribosomes (7–11). We observed that naked 16S and 23S RNAs become highly resistant to RNase I under reconstitution conditions (unpublished data). This observation and earlier reports (12, 13) that, under specified conditions in the presence of ethanol, 16S and 23S RNAs assume shapes resembling those of the 30S and 50S subunits, respectively, when viewed in the electron microscope, led us to test whether naked 16S and 23S RNAs would form a complex similar to that formed by the ribosomal subunits under reconstitution conditions or in the presence of ethanol. Preliminary crosslinking experiments using psoralen indicated that complex formation between the two rRNA species does occur (14). Further evidence for complex formation when the naked 16S and 23S RNAs are mixed under appropriate conditions is presented here.

MATERIALS AND METHODS

Preparations. *E. coli* 70S, 50S, and 30S ribosomes were prepared by ultracentrifugation as described (9). 16S and 23S RNAs were isolated from 30S and 50S ribosomes, respectively, by treatment with phenol in the presence of $NaDodSO_4$, followed by precipitation with ethanol as described (6). The 23S RNA was separated from 5S RNA by filtration through Sephadex G-100 (15). ^{32}P-Labeled 16S and 23S RNAs were prepared from cells grown in ^{32}P-containing medium. All reagents were of analytical grade.

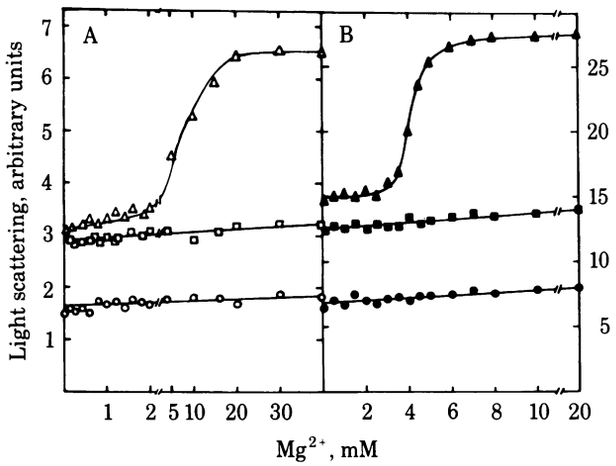

FIG. 1. Association of 16S and 23S RNAs at various Mg^{2+} concentrations as measured by light scattering. (*A*) Association of equimolar amounts of 16S and 23S RNAs. ○, 16S RNA; □, 23S RNA; △, 16S RNA/23S RNA. (*B*) Association of equimolar amounts of 30S and 50S ribosomes. ●, 30S ribosomes; ■, 50S ribosomes; ▲, 30S ribosomes/50S ribosomes.

Assays. *Light scattering.* Solutions of 16S and 23S RNAs were separately dialyzed for 12 hr against 20 mM Tris·HCl, pH 7.5/400 mM KCl containing the desired amount of Mg^{2+}. The two solutions were then mixed in the required proportions and light scattering was measured at an angle of 90° in a 1-ml cuvette in an Aminco Bowman spectrofluorometer. The excitation and emission monochromators were set to 400 nm. Similar measurements were carried out at low salt concentration in the presence of 1.0 M ethanol. For measurement of the association of 30S and 50S subunits in control experiments, each preparation was dialyzed for 24 hr against 20 mM Tris·HCl, pH 7.5/30 mM NH_4Cl/0.1 mM $Mg(OAc)_2$/6 mM 2-mercaptoethanol. Light scattering was measured as described for the RNA mixture.

Sucrose density gradient centrifugation. Aliquots (0.2 ml) of the dialyzed preparations or mixtures of 23S and 16S RNAs in reconstitution buffer (20 mM Tris·HCl, pH 7.5/400 mM KCl/20 mM $MgCl_2$) were layered on 4.8 ml of a 5–20% sucrose gradient in reconstitution buffer. Centrifugation was carried out at 150,000 × *g* for 6 hr in a Zanetzki VAC 601 ultracentrifuge. The fractions were collected from the bottom and the absorbance was measured at 260 nm.

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Composite polyacrylamide/agarose gel electrophoresis. This method could not be used in the presence of high salt because of generation of heavy convection currents. It was used only in the presence of 1.0 M ethanol at low salt concentrations. A mixture of ^{32}P-labeled 16S and 23S RNAs in the presence of 1.0 M ethanol/20 mM $Mg(OAc)_2$/60 mM NH_4OAc, pH 7.5, was subjected to electrophoresis on composite gels containing 2% acrylamide and 0.5% agarose. Both the gel and the electrode buffer contained the same buffer mixture. This procedure was also used for separation of noncomplexed 16S and 23S RNAs but in this case the buffer was 10 mM Tris·HCl, pH 7.5/0.1 M KCl/1 mM EDTA.

RESULTS

Association of 16S and 23S RNAs in Reconstitution Buffer. Equimolar amounts of the two RNA species were mixed in 20 mM Tris·HCl, pH 7.5/400 mM KCl containing various concentrations of Mg^{2+}, and the interaction of the RNAs was measured by light scattering (Fig. 1*A*). The association of the 30S and 50S ribosomes as a function of the Mg^{2+} concentration was also examined in 20 mM Tris·HCl, pH 7.5/30 mM NH_4Cl (Fig. 1*B*). Similar association curves were obtained for the two systems. However, the RNA association required much higher salt (400 vs. 30 mM) and higher Mg^{2+} (20 vs. ≈6 mM) concentrations than the ribosomal subunit association. When the concentration of Mg^{2+} was fixed at 20 mM and that of KCl was varied, maximum association took place at 400 mM KCl (data not shown). Similarly, pH 7.5 was found to be optimum for the RNA complex formation (data not shown).

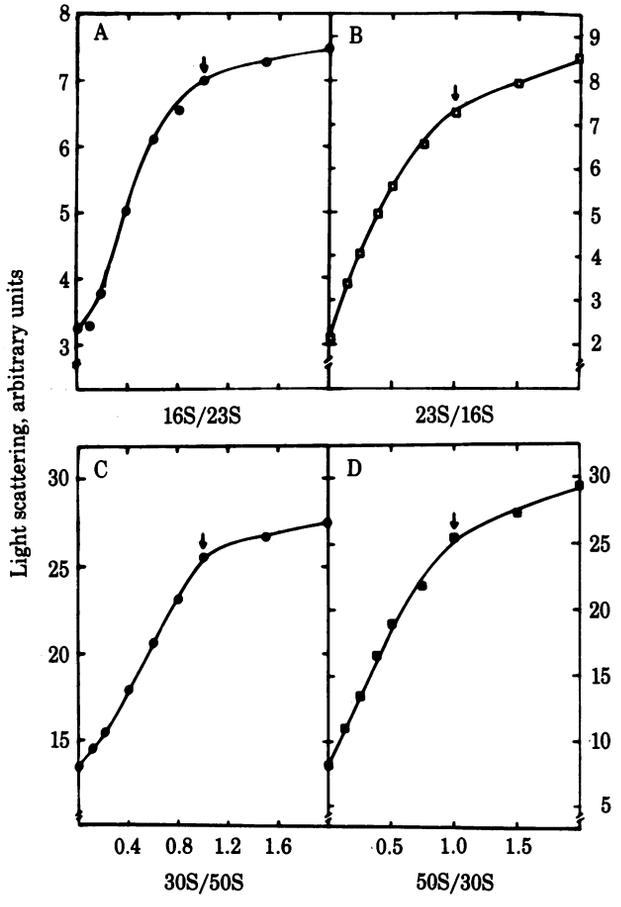

FIG. 2. Stoichiometry of association of 16S and 23S RNAs as measured by light scattering. Light scattering (arbitrary units) is plotted against molar ratio of components. (*A*) Various amounts of 16S RNA were added to a fixed amount of 23S RNA (2 A_{260} units) in reconstitution buffer. (*B*) Various amounts of 23S RNA were added to a fixed amount of 16S RNA (1 A_{260} unit) in reconstitution buffer. (*C*) Various amounts of 30S ribosomes were added to a fixed amount of 50S ribosomes (2 A_{260} units) in association buffer [Tris·HCl, pH 7.5/30 mM NH_4Cl/10 mM $Mg(OAc)_2$]. (*D*) Various amounts of 50S ribosomes were added to a fixed amount of 30S ribosomes (1 A_{260} unit) in association buffer.

Stoichiometry of the Association of 16S and 23S RNAs. *Light scattering.* To determine whether the association of naked RNAs is stoichiometric, as is ribosomal subunit association, two types of experiments were carried out. In one case (Fig. 2*A*), the amount of 23S RNA was kept constant and increasingly large amounts of 16S RNA were added under reconstitution conditions; in the other case (Fig. 2*B*) increasingly large amounts of 23S RNA were added to a fixed amount of 16S RNA. In each case, association was measured by light scattering. For comparison, similar experiments were carried out with 30S and 50S ribosomes (Fig. 2 *C* and *D*). We observed decreases in the slopes of the curves beyond the points (indicated by arrows) at which the pairs were present in equimolar amounts. This was true for the naked RNAs as well as for the ribosomal subunits. The small increase in scattering beyond this point is due to scattering by the species added in excess.

Sucrose density gradient centrifugation. Complex formation between 16S and 23S RNAs was shown directly by this method (Fig. 3), which also allowed direct determination of the stoichiometry of the association reaction. The 16S and 23S RNAs were mixed in various proportions, increasing the amount of one type of RNA with respect to the other. The gradients contained the reconstitution buffer to prevent dissociation during centrifugation. In each case, complex formation was maximum when the two species of RNA were present in equimolar amounts.

Evidence for the Presence of Equimolar Amounts of the 16S and 23S RNAs in the Complex. To confirm that the 16S and 23S RNAs are present in equimolar amounts in the complex, ^{32}P-labeled RNAs were used. Radioactive RNAs were mixed under reconstitution conditions, and the mixture was subjected to sucrose gradient centrifugation as described in Fig. 3. The complex was precipitated with ethanol from the fractions as indicated, and the precipitates were dissolved in 10 mM Tris·HCl,

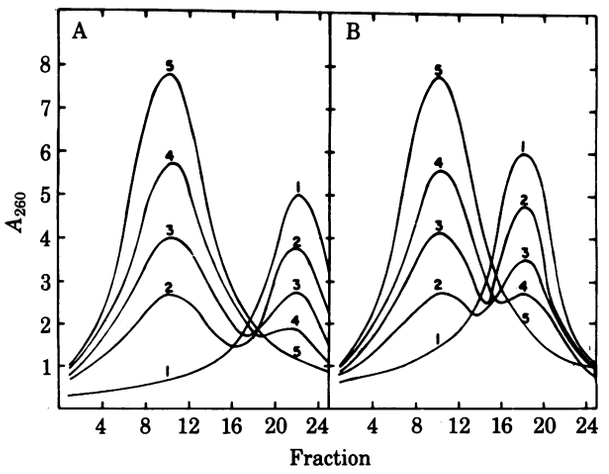

FIG. 3. Stoichiometry of association of 16S and 23S RNAs as measured by sucrose density gradient centrifugation. Various amounts of 23S RNA were added to a fixed amount of 16S RNA (*A*) or various amounts of 16S RNA were added to a fixed amount of 23S RNA (*B*) under reconstitution conditions. (*A*) 23S/16S ratios (mol/mol) were 0 (curve 1), 0.25 (curve 2), 0.50 (curve 3), 0.75 (curve 4), and 1.0 (curve 5). (*B*) 16S/23S ratios (mol/mol) were 0 (curve 1), 0.25 (curve 2), 0.50 (curve 3), 0.75 (curve 4), and 1.00 (curve 5).

pH 7.5/100 mM KCl/1 mM EDTA and dialyzed against the same buffer for 25 hr. Low-salt conditions and EDTA were used to dissociate the complex. Aliquots of the dialyzed solution were subjected to composite polyacrylamide/agarose gel electrophoresis in the same buffer to separate the 16S and 23S RNAs (Fig. 4*A*). A mixture of unassociated 16S and 23S RNAs was also subjected to electrophoresis as a control (Fig. 4*B*). At the end of the run, the gels were sliced and the radioactivity of the slices was assayed in a liquid scintillation counter. From the results in Fig. 4, we concluded that the 16S and 23S RNAs were present in the complex in equimolar amounts.

Association of the 16S and 23S RNAs in the Presence of Ethanol. Vasiliev and co-workers (12, 13) have reported that, under certain ionic conditions and in the presence of 1.0 M ethanol, 16S and 23S RNAs assume shapes similar to those of 30S and 50S subunits, suggesting that, in the presence of ethanol, the RNAs assume conformations similar to those obtaining under reconstitution conditions. It was of interest to determine whether the two RNA molecules would form a complex in the presence of ethanol. Light-scattering measurements of a mixture of 16S and 23S RNAs in the presence of various amounts of ethanol, magnesium acetate, and ammonium acetate showed that maximum association occurred when these components were present at 1 M, 20 mM, and 60 mM, respectively (Fig. 5). The optimum pH for association was 7.5. Thus, it appears that complex formation can take place under low-salt conditions provided ethanol is present at a concentration of 1.0 M. These conditions are similar to those causing the folding of naked 16S and 23S RNAs into the shapes of 30S and 50S ribosomes, respectively (12, 13). Light-scattering and sucrose gradient centrifugation experiments (data not shown) indicated that the 16S·23S RNA complex is formed in a 1:1 ratio also under these conditions. It was not possible to demonstrate formation of the 16S·23S complex by polyacrylamide gel electrophoresis under reconstitution conditions because of the high salt concentration, but this could be done in the presence of ethanol at low salt concentrations. ^{32}P-Labeled 16S and 23S RNAs, either individually or in equimolar mixtures, were subjected to composite polyacrylamide/agarose gel electrophoresis in 20 mM $Mg(OAc)_2$/60 mM NH_4OAc/1.0 M ethanol, pH 7.5. At the end of the run, the gels were sliced and the radioactivity of the slices was determined. The data for all three gels are presented in Fig. 6. When the two species of RNA were mixed in equimolar amounts, none was present in the free state; a new slower moving species was formed, indicating complex formation.

DISCUSSION

The present study may help to elucidate the mechanism of ribosomal subunit association. We have observed that naked 16S

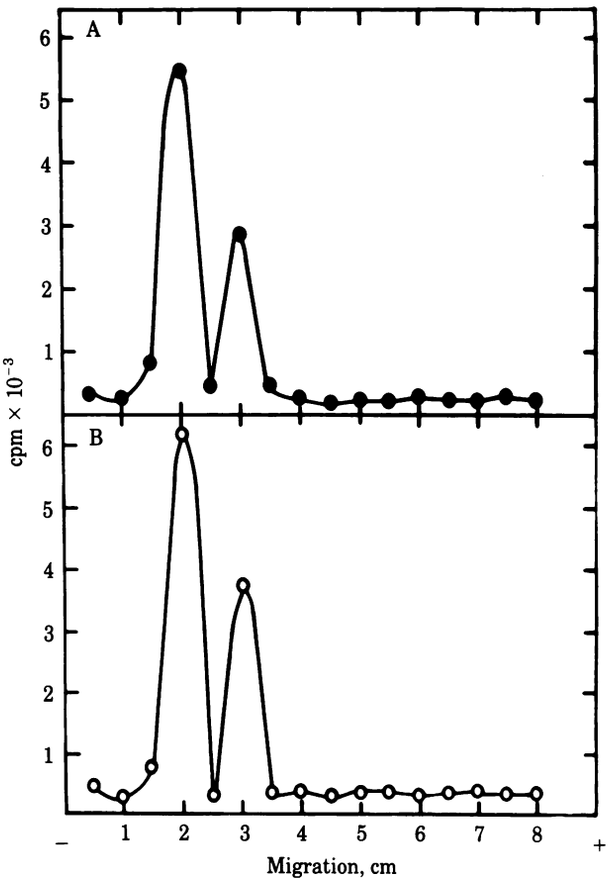

FIG. 4. Evidence for the presence of 16S and 23S RNAs in equimolar amounts in the complex. Five A_{260} units of ^{32}P-labeled 16S RNA (65,000 cpm) and 9.5 A_{260} units of ^{32}P-labeled 23S RNA (128,000 cpm) were mixed in reconstitution buffer and subjected to sucrose gradient centrifugation as described in Fig. 3. The fractions containing the complex were pooled, treated with 2 vol of ethanol, and processed. Aliquots (12,000 cpm) were subjected to composite polyacrylamide/agarose gel electrophoresis. The gel was sliced and the radioactivity of the slices was determined. (*A*) Complex. (*B*) Mixture of RNAs.

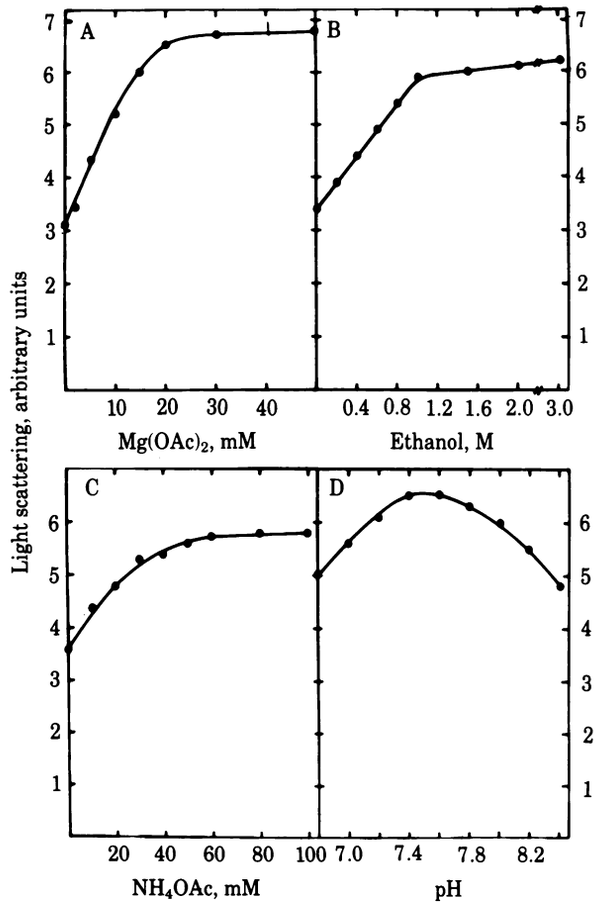

FIG. 5. Optimum conditions for the association of 16S and 23S RNAs in the presence of ethanol. One A_{260} unit of 16S RNA and 1.9 A_{260} units of 23S RNA were mixed in 1 ml of 1 M ethanol/60 mM NH_4OAc containing various amounts of $Mg(OAc)_2$ (*A*), 60 mM NH_4OAc/20 mM $Mg(OAc)_2$ containing various amounts of ethanol (*B*), 20 mM $Mg(OAc)_2$/1 M ethanol containing various amounts of NH_4OAc (*C*), or 1 M ethanol/60 mM NH_4OAc/20 mM $Mg(OAc)_2$ at varying pH values adjusted with NH_4OH. The mixtures were kept at 40°C for 10 min and then at 4°C for 1 hr as described (13).

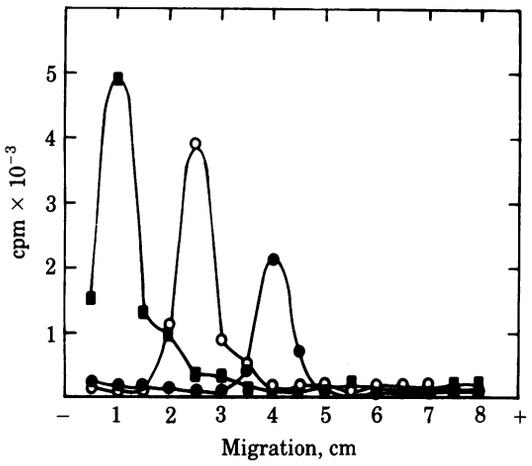

FIG. 6. Evidence for formation of the 16S·23S RNA complex in the presence of ethanol by composite polyacrylamide/agarose gel electrophoresis. An amount (0.3 A_{260} unit) of ^{32}P-labeled 16S RNA (3,000 cpm), 0.57 unit of ^{32}P-labeled 23S RNA (5,700 cpm), and a mixture of the two in 0.05 ml were treated separately with 20 mM $Mg(OAc)_2$/60 mM ammonium acetate/1 M ethanol, pH 7.5. Each sample was subjected to composite polyacrylamide/agarose gel electrophoresis. ●, 16S RNA; ○, 23S RNA; ■, 16S/23S RNA mixture.

and 23S RNAs associate with each other much as do the ribosomal subunits. However, the conditions are not the same; considerably higher salt (400 mM) and Mg^{2+} (20 mM) concentrations are necessary in the former case. Low salt (60 mM) leads to similar association when ethanol is present at a concentration of 1.0 M. The studies on subunit association and dissociation were originally carried out by sucrose density gradient centrifugation although it is well known that hydrostatic pressure developed during ultracentrifugation induces dissociation of the subunits. To avoid this, crosslinking of the two subunits before centrifugation was used (for example, ref. 16). However, the light-scattering method is free of this disadvantage (17). Its main drawback is that it does not permit visualization of the molecular events but presents a gross picture. Our work shows that the two methods are complementary and lead to the same conclusion.

Several types of interactions—e.g., RNA–RNA, RNA–protein, protein–protein—may be involved in the association of the ribosomal subunits. Of these, RNA–RNA interactions have been implicated by several workers (18–23) although direct evidence has so far not been presented. The formation of a complex between 16S and 23S RNAs reported here also suggests that RNA–RNA interaction may play a major role in ribosomal subunit association. Although the details of the 23S–16S rRNA complex formation remain to be worked out, the nature of the association does not seem to be very different from that observed with the ribosomal subunits, particularly when the amount of magnesium required for the association reactions is considered. This can be calculated from the results presented in Fig. 1 by plotting log [70S]/[50S][30S] or log [16S–23S]/[16S][23S] against log [Mg^{2+}]. Five molecules of Mg^{2+} appear to be involved with naked RNAs while six molecules are required for association of the subunits. Several other questions raised by this study remain to be answered. For example, is there any involvement of 5S RNA in the association? Preliminary results (not presented) indicate that 5S RNA alone, in the absence of 5S RNA-binding proteins, is not incorporated into the complex. Another question is whether IF-3 can interfere with the association of 16S and 23S RNAs as in the case of ribosomal subunits (see ref. 24). Some preliminary experiments suggest that this may be so. It also has to be established whether the recognition between 23S and 16S RNA is based on Watson–Crick base pairing or on some other mechanism. It has been possible to crosslink the two RNA molecules present in the complex (14) and this may provide an answer. The observations recorded here could also have some evolutionary significance.

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