Origin of Informational Polymers

DIFFERENTIAL STABILITY OF PHOSPHOESTER BONDS IN RIBOMONOMERS AND RIBIOLOGOMERS*

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We have measured the stabilities of the bonds that are critical for determining the half-life of ribonucleotides and the β-glycosidic and 3′- and 5′-phosphoester bonds. Stabilities were measured under a wide range of temperatures and water/formamide ratios. The stability of phosphodiester bonds in oligoribonucleotides was determined in the same environments. The comparison of bond stabilities in the monomer versus the polymer forms of the ribo compounds revealed that physico-chemical conditions exist in which polymerization is thermodynamically favored. These conditions were compared with those determining a similar behavior for 2′-deoxyribonucleosides, deoxyribonucleotides, and deoxyribonucleotides and were shown to profoundly differ. The implications of these facts on the origin of informational polymers are discussed.

Minimal requirements for informational polymers to have originated under prebiotic conditions are a unitary chemical frame for the synthesis of nucleobases and a favorable thermodynamic energy balance for the polymerization of nucleotides to oligonucleotides.

A Formamide-based Unitary Chemistry—To gather into a single reaction milieu all the precursors necessary for the assembly of the first informational polymers, a unitary chemical frame is needed. A series of observations suggest that formamide (NH₂CHO) chemistry might have provided such a frame.

To summarize the evidence: the one-carbon compound formamide is a highly versatile building block for the syntheses of all the necessary precursor nucleic bases. We have reported (Refs. 1–4 and references therein) the synthesis of purine, N⁹-formylpurine, adenine, N⁶,N⁶-diformyladenosine, hypoxanthine, cytosine, hydroxypyrimidine, 4(3H)-pyrimidinone, uracil, 5,6-dihydrouracil, thymine, 5-hydroxymethyluracil, AICA (5-aminoimidazole-4-carboxamide), and urea. These compounds are obtained from formamide heated in the presence of simple inorganic catalysts such as silica, alumina, zeolites, CaCO₃, TiO₂, common clays, kaolin, montmorillonites, olivines, and cosmic dust analogues of olivines. In all cases and for each catalyst several compounds were simultaneously formed, as summarized in Ref. 4.

The richest yields were obtained in the presence of clays, TiO₂, and cosmic dust analogues. Guanine is conspicuously absent, but a possible efficient substitute in base pairing might have been provided by hypoxanthine. Notably, formamide also yields purine acylnucleosides through a formose-like condensation of N-formyl derivatives (2), providing a solution to the long-standing problem of the lack of reactivity between nucleobases and ribose or 2′-deoxyribose. In addition, formamide is an efficient activator of nucleoside transphosphorylation (Refs. 5–7), further supporting its possible relevance in the prebiotic polymerization reactions that did lead to pre-genetic informational macromolecules.

Formamide is easily formed by hydrolysis of HCN and is stable in liquid form over a wide range of temperatures (2.5–210 °C). This property favors its concentration from dilute aqueous solutions by moderate heating. These physico-chemical attributes and its presence along with that of H₂O and HCN in planets, comets, asteroids, and in the interstellar space (8) support its possible prebiotic role.

A hypothetical, but plausible and simple, scenario could thus consist of the following: in the presence of a varied (perhaps complete) panel of nucleic acid precursors dissolved in a water/formamide solution at moderately high temperature and in the presence of a phosphate donor, formamide-based trans-phosphorylation reactions would lead to the formation of oligomers and kick start a molecular evolutionary process. We are not dealing here in any further detail with such a highly hypothetical and fragmentarily outlined synthetic process, our intention being limited to the indication of its chemical plausibility.

The Thermodynamic Problem—In the origin of the informational polymers, their survival as macromolecules is even more problematic than the polymerization process itself. Do physico-chemical conditions exist, providing kinetic and/or thermodynamic advantage to the otherwise intrinsically more unstable polymeric form over the monomer?

We focus here on RNA and describe a systematic comparison of the stability of the phosphoester bonds in the precursor monomers versus the stability of the same bonds when present in RNA, under a wide range of temperatures and water/formamide ratios. Such comparison was lacking and provides information directly relevant to the monomer versus polymer bias. The stability of the β-glycosidic bond in the same set of conditions was also determined.

A parallel analysis was recently performed (9) comparing the corresponding stability properties for 2′-deoxyribonucleosides, deoxyribonucleosides, and deoxyribonucleotides. Thus, a direct comparison between relative bond stabilities in RNA and DNA is here for the first time provided under comparable conditions.

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EXPERIMENTAL PROCEDURES

Materials

Adenine, ribose, ribose 5'-monophosphate, adenosine, adenosine 5'-monophosphate (5'-AMP), and adenosine 3'-monophosphate (3'-AMP) were from Sigma Aldrich, analytical grade.

The degradation of RNA in water, in formamide, and in formamide-containing aqueous solutions was studied on P1 RNA. This RNA has the sequence 5'—GGAAACGUUCCUUGGGAG-3', was purchased from RNATEC, and was kindly provided by E. Caffarelli (10, 11).

Methods

HPLC Analysis—Samples were resuspended at a final concentration of 1 mg/ml in water or in the appropriate formamide reaction medium (usually in 0.5–1.0 ml). Temperatures and incubation times are indicated. 10-μl aliquots of the reaction mixtures were diluted to a final concentration of 50% formamide in a final volume of 20 μl and injected into a Supelcosil™ LC-18-S 5-μm HPLC column (Supelco) 15 cm × 4.6 mm. Elution was performed at a flow rate of 2 ml/min at room temperature with methanol:30 mM ammonium phosphate, pH 5.3 (2.5:97.5), UV 254 nm, pressure 1.5 atmosphere, on an HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with real samples.

RNA Preparation and Labeling; RNA 5'-Labeling—10 μmol P1 RNA were labeled with [γ-32P]ATP using polynucleotide kinase (Roche Applied Science). The oligo was then purified on a 16% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide). For these methods, see also Ref. 3.

Half-lives of the Bonds in the Ribooligonucleotide—For oligonucleotides, the half-lives of the phosphoester bonds were determined from the rate of disappearance of the band representing the intact 20-mer molecule. Cleavage of RNA normally requires participation of the 2'-OH group as an internal nucleophile (12) by two “nucleophilic cleavage” events: the trans-esterification and hydrolysis reactions (Fig. 1). During the trans-esterification (pathway a), the 2'-OH nucleophile attacks on tetrahedral phosphorus (V) affording a 2',3'-cyclic mono-phosphate I, which in turn can be hydrolyzed to a mixture of 3'- and 2'-phosphate monoester II and III, respectively (pathway b). Both steps can be catalyzed by a large range of chemical species, including protons, hydroxide, nitrogen derivatives, and metal ions. On the basis of this known mechanism two operative assumptions were made. 1) It was assumed that the cleavage of the 3'-phosphoester bond is largely more effective than the cleavage of the 5'-one and that for practical calculation purposes the 5'-phosphoester bond is not cleaved. In support, the cleavage of the 5' terminally labeled RNA molecule only shows the standard cleavage of the 3'-bond (see Fig. 6), whereas the double bands typically produced by 3'- and 5'-cleavages occurring in the same population of molecules were never detected. This contrasts with DNA (9), where under several conditions both cleavages do occur, giving rise to the diagnostic double bands. 2) In the ribooligonucleotide studied here, the breakage of the phosphodiester chain is not sequence biased under the conditions used. This sequence may have, like many other RNAs, preferential breakage sites (10, 11) that require special defined conditions to be cleaved. Outside the special cleavage conditions this RNA is stable (see Fig. 2 of Ref. 10). These conditions are well characterized (10, 11) and are different from the ones here.

The half-life of the oligonucleotide was determined with standard graphical procedure from plots of the % disappearance of the intact 20-mer molecules. Given that one disappearing molecule represents one cleavage, and given the two assumptions reported (no 5'-cleavage, no sequence bias), the half-life of a 3'-phosphoester bond in the ribooligonucleotide is given by the half-life of the oligonucleotide × 19 (that is: the number of 3'-phosphoester bonds in the 20-mer).

RESULTS

The Stability of the β-Glycosidic and of the 3'- and 5'-Phosphoester Bonds in Nucleosides and Nucleotides

The β-Glycosidic Bond—The stability of the β-glycosidic bond of nucleosides in water was determined in early studies under several conditions (13). We describe here the effect of formamide on the stability of this bond. Measurements were performed as a function of formamide concentration in water and of temperature. In addition to its interest for the stability of this bond, determination of the stability of this bond is a prerequisite for the analysis of the stability of phosphoester bonds in nucleotides and in oligonucleotides.

Fig. 2 shows the high pressure liquid chromatography (HPLC)3 analysis of the degradation of adenosine (% ordinate) as a function of time (abscissa) and of the concentration of formamide in the aqueous medium (from 0 to 100%, as indicated in each panel). The upper panel on the right side provides the interpretation key. The red letters indicate the starting compound, in this case ribose (R) bound to the base (B) adenine. The cleavage (arrow) of the glycosidic bond (red) yields ribose (R, black), which is not detected under our conditions, and adenine (B, green). The same color code is used in the plots of the following figures. Thus, the disappearance of adenosine (red line) is accompanied by the

3 The abbreviation used is: HPLC, high pressure liquid chromatography.
appearance of adenine (green line). For the nucleoside the process is slow.

In the actual HPLC experimental layout, no peaks other than those of adenosine and adenine are observed. Thus, no other cleavage than that of the β-glycosidic bond occurs under this range of conditions. The half-life of this bond at 90 °C in water is >5 × 10^5 min. Increasing concentrations of formamide first decrease and then (>25%) increase the stability of this bond. Fig. 5 shows this double effect exerted by increasing formamide on the stability of the β-glycosidic bond. Fig. 5 also shows the t_{1/2} of the same bond in both 5'- and 3'-adenosine monophosphates (these latter data are from Figs. 3 and 4, respectively, see below). Interestingly, in the presence of a phosphate group in both 3'- and (less so) in the 5'- position, the half-life of the β-glycosidic bond is strongly decreased in water and it is progressively protected by increasing formamide concentrations. The t_{1/2} of the β-glycosidic bond in H_2O is 0.14 × 10^5 min for the 3'-AMP and 0.21 × 10^5 min for the 5'-AMP (see legend to Fig. 5). At formamide >33% the high stability (>5 × 10^5 min) of this bond is re-attained. These effects are markedly sensitive to temperature (Fig. 5, middle and lower panels).

The 3’-Phosphoester Bond—The degradation rates of the 3’-AMP were analyzed as a function of both temperature and formamide concentrations (Fig. 3, the analysis is shown at 90 °C). The disappearance of the 3’-AMP (blue) in water (upper left panel) is matched by the appearance of the nucleoside (red), thus being primarily caused by the cleavage of the 3’-phosphoester bond. In water the β-glycosidic bond is cleaved with a slightly slower kinetics, and the corresponding product (adenine, green) appears with a slower kinetics. Eventually, all the nucleoside is cleaved into sugar (not detected) and adenine. The presence of low concentrations of formamide (starting at 3%) markedly protects the β-glycosidic bond (green line), allowing almost exclusively the cleavage of the phosphoester (red line).

The 5’-Phosphoester Bond—The same analysis was performed on 5’-AMP. The results of the analysis at 90 °C are reported in Fig. 4. The overall trend is similar to that of 3’-AMP, with the notable exception of the behavior in water (upper left panel). Under these conditions the β-glycosidic bond (green line) is cleaved faster than the phosphoester bond (red line). Also, for the 5’-AMP the protective effect exerted by formamide is stronger and starts at low concentration. The half-lives of both the 3’- and 5’-phosphoester bonds as a function of increasing formamide at different temperatures are plotted in Fig. 7.

Summarizing the numerous effects exerted by water/formamide and temperature on the stability of the β-glycosidic and the phosphate bonds in the nucleoside and in its 3’- and 5’-phosphate forms, we observe for the β-glycosidic bond: (i) high stability in water (>5 × 10^5 min) for the nucleoside, (ii) enhanced cleavage by low formamide/water ratios (<33%) versus protection by higher ones (≥33%) (Fig. 5), and (iii) enhanced instability exerted by the presence of a phosphate group, especially so in water (Fig. 5). This latter effect is contrary to what is observed in 5’-deoxymonomers, where the phosphate group exerts a strong protective effect on the β-glycosidic bond in water (Fig. 9 in Ref. 9).

For the phosphoester bonds we observed (Fig. 7) an overall instability (higher for the 3’- than for the 5’-phosphoester bond) and a substantial lack of effects by formamide, contrasting with the marked protection exerted by formamide on the β-glycosidic bond. The comparison of this set of properties with those defined in 2’-deoxymonomers is under “Discussion.”

The Half-life of the 3’-Phosphoester Bond in a Ribooligonucleotide—The 5’-labeled 20-mer ribooligonucleotide described under “Materials” was treated at various temperatures for the indicated times (Fig. 6) in water or in the presence of 1.5, 3, 25, or 100% formamide. Fig. 6 shows one example of the degradation progression observed. The kinetics of degradation of the ribooligonucleotide is calculated from the disappearance of the full-sized molecule (top band) (see “Methods”). Fig. 6 shows that, as a general trend, increasing formamide concentration first increases and then protects RNA from the hydrolytic process. Fig. 7 shows the quantitative evaluation of this type of degradation experiment, performed at 30, 70, and 90 °C (left, middle, and right panels, as indicated) for the appropriate range of times: lower temperatures required longer reaction times. Each
panel shows the half-lives of the 3′-phosphoester bond determined from digestions similar to those reported in Fig. 6. The half-lives of the 5′- and 3′-phosphoester bonds, determined from the HPLC measurements described in Figs. 3 and 4, respectively, are also reported.

The results in Fig. 7 show that in water and at high formamide concentrations at 70° and 90 °C the average 3′-phosphoester bond in the oligonucleotide is more stable than the corresponding one in the nucleotide; the 5′-phosphoester in the oligo is so stable that its cleavage could not be measured (see “Methods” and “Discussion”). The 5′-phosphoester bond in the nucleotide is also always more stable than the 3′-one. The gray shade indicates the area of polymer-higher-than-monomer 3′-phosphoester bond stability.

FIGURE 3. HPLC analysis of the degradation kinetics of 3′-monophosphate adenosine. Color code as above; the complete 3′-AMP molecule is indicated in blue.

FIGURE 4. HPLC analysis of the degradation kinetics of 5′-monophosphate adenosine, as in Figs. 2 and 3.

FIGURE 5. The stability of the β-glycosidic bond in adenosine, in 3′-AMP, and in 5′-AMP as a function of formamide concentration. The half-lives (min × 10^6) are calculated from the experiments reported in Figs. 2 (adenosine), 3 (3′-AMP), and 4 (5′-AMP). The three panels show results obtained at the indicated temperatures of 90 °C (upper, data from Figs. 2–4), 70 °C (middle, HPLC data not shown), and 30 °C (lower, HPLC data not shown). The numerical values of the lowest data points (which are not graphically resolved) are 0.21 × 10^6 (5′-AMP at 90 °C), 0.14 × 10^6 (3′-AMP at 90 °C), 0.60 × 10^6 (5′-AMP at 70 °C), and 0.75 × 10^6 (3′-AMP at 70 °C).
**Bond Stabilities in RNA and Its Precursors**

**DISCUSSION**

We have analyzed the stabilities of the bonds that are more relevant for the persistence of the ribonucleic acid as a polymer, the \( \beta \)-glycosidic and the \( 3' \)- and \( 5' \)-phosphoester ones. The stability of each bond markedly varied depending on the molecular context in which it was embedded. In addition, differential variations of the bond stabilities were observed as a function of the physical (namely temperature) and chemical (namely water and/or formamide) environment.

The effects of the presence of formamide were studied because of the reported synthesis in its presence of a complete set of nucleic acid precursors and of its trans-phosphorylating activity. The hypothetical scenario for the synthesis of the pre-genetic nucleic polymers would thus simply consist of formamide-based syntheses in the presence of the appropriate combination of catalysts (1–4) and of the appropriate phosphate donor. This scenario, however hypothetical, is chemically plausible and justifies the stability analyses reported here. The “warm little pond” imagined by Darwin (14) as the original cradle would in this chemical system contain also formamide. Out of an origin-of-informational-polymers frame of interest the varying stabilities of the \( 3' \)- and the \( 5' \)-phosphoester bonds as a function of the molecular context are relevant per se. We have limited our approach to the phenomenological aspects of the problem, refraining for the moment from detailed chemical mechanistic stud-

![FIGURE 6. Degradation kinetics of the \( 5' \)-labeled P1 RNA as a function of the formamide concentration reported on top of each panel. Samples were treated (see “Methods”) for the indicated time (min) at 70°C. A fully run panel is shown only for the 100% water. Only the full-length front is shown for the others.](image6)

![FIGURE 7. Summary comparison of the stability of the phosphoester bonds in the polymer and in the monomers. The half-life (min \( \times \) 10\(^5\)) of the \( 3' \)-phosphoester bond in RNA is compared with that of the same bond in \( 3' \)-AMP and that of the phosphoester bond in \( 5' \)-AMP as a function of the formamide concentrations and of the temperatures reported. RNA data are from experiments similar to those reported in Fig. 6. Nucleotide data are from Figs. 3 (\( 3' \)-AMP) and 4 (\( 5' \)-AMP). For the meaning of the gray-shaded area, see “Results.”](image7)

ies. Three sets of comparisons highlight the relevant correlations found, as follows.

**Comparison of the Bond Stabilities in Ribo- and in 2'-Deoxyribo-Nucleosides and Nucleotides**—The comparison of the bond stabilities in ribo- and in 2'-deoxyribo- nucleosides and nucleotides (Table 1) shows the following.

1) The \( \beta \)-glycosidic bond is more stable in the ribo- than in the 2'-deoxyribonucleosides by two orders of magnitude. This reactivity is in accordance with previous results on the stability of the \( \beta \)-glycosidic bond in nucleosides and 2'-deoxyribonucleosides under acidic catalysis. In this case, electron-withdrawing substituents at the 2'-position of the carbohydrate moiety, like the 2'-OH group in ribose, lowered the reaction rate of the hydrolysis (15). The presence of the phosphate group remarkably causes diverging effects in the 2'-deoxyribo (stability increase) relative to the ribo (stability decrease) derivatives. This effect is strong (compare Fig. 9A of Ref. 9 and Figs. 2–4 of this report).

The \( \beta \)-glycosidic bond is destabilized by low concentrations of formamide (up to \( \sim \) 25% weight/volume) and then at high concentration is stabilized again. This effect is observed for the 2'-deoxyribonucleoside, for the 2'-deoxyribonucleotide, and for the ribonucleoside, whereas for the ribonucleotide the presence of formamide strongly stabilizes the \( \beta \)-glycosidic bond starting from the lowest concentration tested (3% weight/volume) (Figs. 3 and 4).

The protective effect exerted by formamide is probably due to the formation of an extensive and highly organized hydrogen bond network shielding the removal of nucleobases (9, 16, 17). Because of the high directionality of the hydrogen bond, the position of the phosphate moiety (\( 5' \)-versus \( 3' \)-phosphate) is also a relevant stereo electronic parameter to be considered, \( 5' \)-AMP being more stable than \( 3' \)-AMP. Similar results were obtained in the case of \( 5' \)-deoxy AMP and \( 3' \)-deoxy AMP.

2) The phosphoester bonds are more stable in the 2'-deoxyribonucleotides than in the ribonucleotides, more markedly so for the \( 5' \)- (one order of magnitude) than for the \( 3' \)-one (2–3-fold). In accordance with a general base-catalyzed cleavage mechanism, the weakly basic formamide decreases the stability of the phosphoester bonds in all cases (18). This effect is particularly strong for \( 5' \)-deoxy AMP (Fig. 9B of Ref. 9).

**Comparison of Bond Stabilities in Ribomononucleotides and Ribooligonucleotides**—The comparison of bond stabilities in ribomononucleotides and ribooligonucleotides (Fig. 7) shows that in water and in a large area corresponding to high formamide concentrations, the \( 3' \)-phosphoester bond is more stable in the polymer than in the monomer. Because this is the weak bond of the RNA phosphodiester chain, the conditions
identified (shaded in gray) define the physical frame in which the ribooligonucleotide has a thermodynamic advantage over the monomer. The other conditions tested (varying H$_2$O/formamide ratios, higher and lower temperatures) all appear to favor the persistence of the monomer as an unpolymerized unit.

**Comparison of Bond Stabilities in Ribooligonucleotides and 2'-Deoxyribooligonucleotides**—We compared (Fig. 8) the half-lives of the phosphodiester bonds: the 5'- and 3'-bonds in 2'-deoxyribooligonucleotides and the 3'-bond in ribooligonucleotides. The ribo data are the same as those plotted in Fig. 7 and are here reported for the sake of comparison. The 2'-deoxyribo data are from Ref. 9 (for the experiment at 90 °C) and from similar experiments performed with exactly the same temperature (data not shown) exactly as for the ones at 90 °C. For the meaning of the gray-shaded area, see “Results.”

**TABLE 1**

**Comparison of bond stabilities in ribomononers and deoxyribomononers**

| Bond Type          | Deoxyribonucleoside | Deoxyribonucleotide | Ribonucleoside | Ribonucleotide |
|--------------------|---------------------|---------------------|----------------|---------------|
| $\beta$-glycosidic |                      |                     |                |               |
| H$_2$O             | $10 \times 10^3$    | ↓ (low), ↑ (high)   | $>5 \times 10^3$ | ↓ (both 3' and 5') |
| Formamide          |                     |                     |                |               |
| Phosphodiester 5'  | $>25 \times 10^3$   | ↓                   |                | $2.0 \times 10^3$ |
| H$_2$O             |                     |                     |                |               |
| Formamide          |                     |                     |                |               |
| Phosphodiester 3'  | $2.5 \times 10^3$   | ↓                   | $0.9 \times 10^3$ |               |
| H$_2$O             |                     |                     |                |               |

**FIGURE 8. Summary comparison of the stability of the phosphoester bonds in RNA and DNA.**

The half-life (min $\times 10^3$) of the 3'-phosphoester bond in RNA (same data as in Fig. 7) is compared with the half-lives of the 3'- and 5'-phosphoester bonds in DNA, obtained at the temperatures and the formamide concentrations indicated. The DNA data at 90 °C are from Ref. 9. The data at 30 and 70 °C are from experiments performed at the indicated temperature (data not shown) exactly as for the ones at 90 °C. For the meaning of the gray-shaded area, see “Results.”

**Bond Stabilities in RNA and Its Precursors**

Starting from the consideration that the condensation reactions are not thermodynamically spontaneous in dilute aqueous solution or even at moderate water activities, two possible solutions have been proposed for their occurrence (31). The first is the mechanism used by contemporary organisms, the activation of monomers by phosphorylation. The second is the evasion from the unfavorable Gibbs free-energy change ($\Delta G^\circ$) by reducing the water activity. As for the first, the fact that phosphorylated monomers played a resolutory role in prebiotic polymerization remains a unique property of RNA at present and a support for RNA being an older information carrier than DNA.

Codogenic structural and catalytic abilities are not absolute discriminating factors in deciding whether a “DNA world” existed before or after, or whether since the beginning it co-evolved, with the RNA world. Ignoring the physico-chemical conditions in which initial informational polymers formed, survived, and evolved does prevent an experimentally testable discrimination.

A fourth property of the polymeric systems that might provide information on the origin of informational polymers and on the who-came-first problem is the ensemble of thermodynamic and kinetics considerations pertaining to polymerization process and polymers stability. Let us dub this property with a collective name: persistence. This topic is critically examined and brought to propositive conclusions in Ref. 31.
Bond Stabilities in RNA and Its Precursors

The idea of a primeval soup is therefore not thermodynamically sound.

If syntheses of precursor monomers had occurred in an aqueous solution, the considerations reported above imply the necessity of a process involving dehydration and subsequent concentration. Given that the conditions needed for the syntheses of the first polynucleotides (dry chemistry) and for their template copying (solution chemistry) are essentially antithetical, repeated cycles of hydration and dehydration would have been required.

These thermodynamic considerations help in delineating the physico-chemical initial scenario. Small bodies of water (i.e. aqueous droplets trapped in crevices in rocks) subject to periodic evaporation and re-hydration were suggested (31) as initial reaction milieu. This condition, the considerations reported above imply the necessity of a process involving dehydration and subsequent concentration. Given that the thermodynamic considerations help in delineating the physico-chemical initial scenario. Small bodies of water (i.e. aqueous droplets trapped in crevices in rocks) subject to periodic evaporation and re-hydration were suggested (31) as initial reaction milieu.

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