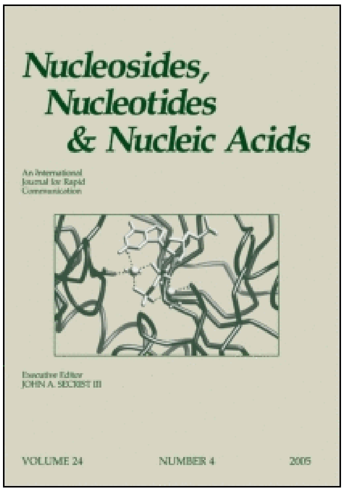

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Imidazole Ring Opening of 7-Methylguanosine at Physiological pH

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IMIDAZOLE RING OPENING OF 7-METHYLGUANOSINE AT PHYSIOLOGICAL pH.

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ABSTRACT

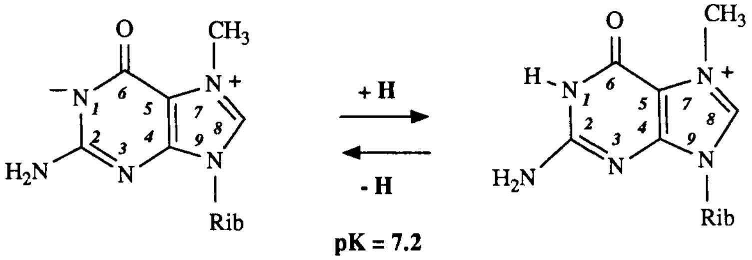
It is shown by carbon-13 NMR that the only product of the imidazole ring-opening of 7-methylguanosine at physiological pH is 2-amino-6-hydroxy-5-N-methylformamido-4-(N-β-ribofuranosylamino)pyrimidine and that, contrary to previous results, the hydrolysis of the glycosylic bond at pH=7.2 is a much slower process than the ring opening. The ring opened formamidopyrimidine derivative is a very mobile molecule existing under different conformations depending on the solvent, and in water it is capable of giving different kinds of aggregates. Possible biochemical implications of these results are discussed.

The structure and the biological role of the ring opened derivatives of 7-methylguanosine (hereafter referred to as m7Guo) has long been, and still is, a matter of interest[1-3]. The reason for this lies mainly in the fact that: *i)* 7mGuo is a component of the 5'- terminal region, i.e. of the 'cap' structure of several eukaryotic mRNAs[4-6] *ii)* the N7 atom of guanine is the principal (>60%) methylation site of DNA by carcinogenic agents such as dimethylsulphate or N-methyl-N-nitrosourea, both in vitro and in vivo[7-9] *iii)* the ring opened derivatives of m7Guo are inhibitors of the in vitro synthesis of DNA as they block the chain elongation[10]. The DNA lesions formed by opening of the imidazole ring are much more persistent than m7Guo lesions, which are removed efficiently from DNA[11-13]. However, the cellular mechanism by which m7Guo may be converted to the ring opened formamidopyrimidine derivative has not yet been elucidated[11]. In the last few years several papers, with conflicting results, have been dedicated to the question of the presence of ring opened derivatives of m7Guo in the DNA of rats given methylating carcinogenic agents[14-16].

In the early 60's it was reported[1] that in moderately basic aqueous solution m7Guo undergoes cleavage of the imidazole ring with formation of 2-amino-6-hydroxy-5-N-methylformamido-4-(N-β-ribofuranosylamino)pyrimidine. Ten years later, it was found[7] that the product of ring opening of m7Guo appeared homogeneous on paper chromatography but, when this material was eluted into water and chromatographed, two different peaks were observed, both having the same absorption spectrum. Later, column chromatography and mass spectrometry data indicated that the ring opened m7Guo consisted of several species with the predominance of one formylated and one deformylated pyrimidine derivative[2,17]. Further results in contrast to these ones were published more recently[3].

So far, most of the studies on the ring opening of m7Guo have been carried out in strongly basic conditions (generally, NaOH 0.2M), required by the fact that commercial samples containing more than fifty percent of the protonated form (SCHEME 1) of m7Guo[18] were employed. However, we have shown that the pure deprotonated form of m7Guo can be obtained by air oxidation of 7,8-dihydro-7-methylguanosine[19]. Thus, we started a carbon-13 magnetic resonance investigation, firstly, to check whether spontaneous ring fission of m7Guo may occur at physiological pH, and secondly, if this is the case, to establish the number and the structure of the ring opened derivatives. We report here our results.

SCHEME 1



7-methylguanosine

EXPERIMENTAL PROCEDURES

The starting materials was air oxidized 7,8-dihydro-7-methylguanosine (Fluka). 0.1 M and 0.5 M solutions of these compounds in phosphate saline buffered solution (pH = 7.2), NaOH 0.2M or wet dimethylsulfoxide were prepared in 5 mm NMR tubes and monitored by carbon-13 NMR at selected time intervals.

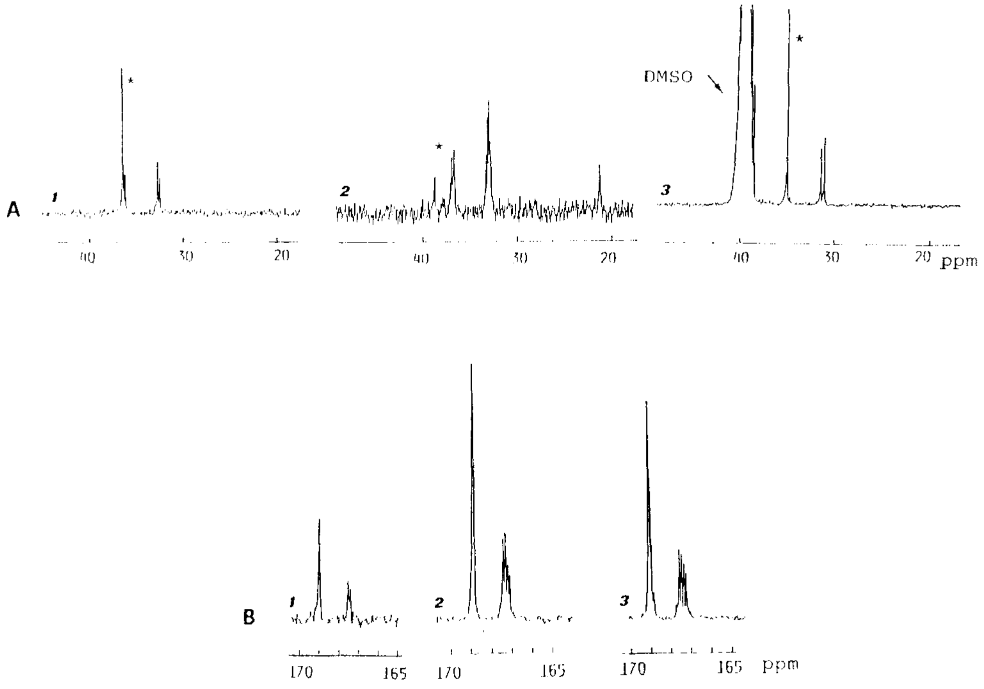


FIGURE 1

A) *N-CH3 region of the 75.4 MHz carbon-13 NMR spectrum of a 0.1 M solution of 7-methylguanosine at pH = 7.2, monitored after 25 hours (1), in NaOH 0.2 M after 4 hours (2) and in dimethylsulfoxide after one month (3). (*) N-CH3 resonance of the starting material.* **B)** *N-CHO region of the 75.4 MHz carbon-13 NMR spectrum of a 0.5 M solution of 7-methylguanosine at pH = 7.2 after 7 hours (1), 24 hours (2) and 47 hours (3). The slow formation of aggregates is shown by the appearance of 'structure' lines.*

The assignement of the carbon-13 NMR signals was based on proton-carbon correlated NMR spectra and on the comparison with literature data[20]. The % of m7Guo ring fission was estimated by integration of the C2' region of the spectrum, since C2' is the sharpest and tallest peak of the spectrum, in both m7Guo and r.o. m7Guo.

The reference for the carbon-13 spectra was internal TMS in DMSO and internal dioxane in water (conversion factor: δ^{TMS} (diox) = 67.4 ppm). For the nitrogen-15 spectra the reference was an external 1 M solution of HNO_3.

All spectra were obtained with a Bruker CXP-300 MHz spectrometer at 75.4 MHz for carbon-13 and at 30.408 MHz for nitrogen-15 (10 mm tubes and a dedicated probe).

TABLE 1.

Carbon-13 NMR chemical shifts[a] of deprotonated 7-methylguanosine (m7Guo) in water and DMSO, and of the ring opened conformers (r.o.) in the same solvents

	C2	C4	C5	C6	C8	CH_3
			H_2O, phosphate buffer, pH=7.2			
m7Guo[b]	162.8	149.9	110.0	163.9	135.2	36.6
r.o.[c]	163.5	155.5	97.4	161.6	169.0[d]	36.3, 36.4 *(Z-form)*
R=Rib.	163.2	155.9	95.0	161.6	167.5, 167.4	32.8, 32.5 *(E-form)*
r.o.	162.1	155.4	96.5	163.8	169.3	36.3
R=H	162.1	155.4	93.1	163.8	167.6	32.5
			DMSO			
m7Guo[e]	162.9	149.1	108.6	162.4	132.2	35.0
r.o.[f] R=Rib.	160.0,160.2	154.5	95.2,95.1	161.5	165.5,165.6	31.1,31.5 *(E-form)*
r.o. R=H	160.6	154.3	94.4	161.7	165.3	31.1[e]

(a) δ(TMS), ppm; (b) Sugar residue: 90.6(C1'); 86.6(C4'); 74.8(C3'); 70.2(C2'); 61.7(C5'); (c) Sugar residue: 83.0, 83.2(C1'); 84.8, 84.8(C4'); 73.1,73.2(C3'); 70.1, 70.3(C2'); 61.8, 61.9(C5'); (d) double intensity; (e) Sugar residue: 90.7(C1'), 86.9(C4'), 74.8(C3'), 70.9(C2'), 61.9(C5'); (f) Sugar residue: 85.9, 86.0(C1'); 84.0, 84.1(C4'); 74.5,74.5(C3'); 71.1, 71.1(C2'); 62.4, 62.4(C5').

RESULTS

The results - summarized in FIGURE 1 and in TABLE 1 - were the following:

1) At physiological pH the half time for a pseudo first-order ring opening reaction of a 0.1 M solution of m7Guo at 25°C is $\tau_{1/2}$ = 85 hours. No traces of free ribose were found in the spectrum one month after the preparation of the sample, indicating that, contrary to previous assumptions[21], the hydrolysis of the glycosylic bond is a much slower reaction than the ring opening.

The ring opening at physiological pH is a clean reaction which gives only a single product, 2-amino-6-hydroxy-5-N-methylformamido-4-(N-β-ribofuranosylamino)pyrimidine (SCHEME 2 for R=Ribose). However, the ring opened derivative is a very mobile molecule which in water exists in *four* different conformations in slow equilibrium in the NMR time scale, so that four separate sets of signals are observed in the carbon-13 spectrum (TABLE 1 and FIGURE 1A). Moreover, in a few hours all conformers undergo a further dynamic process which manifests itself through the appearance, near each of the carbon-13 peaks, of 'structure lines', i.e. of lines having chemical shifts which differ by only a few Hz from those of the original ones (whose intensities decrease) and which are clearly relative to the same kind of carbon atoms in slightly different electronical environments (FIGURE 1B). The phenomenon is typical of the formation of different kinds of aggregates[22, 23] .

2) In NaOH 0.2M the ring opening reaction is much faster than at physiological pH. For a 0.1M solution of m7Guo in NaOH 0.2 M at 25°C after four hours (the time to get a reasonable signal to noise ratio of the carbon-13 spectrum) 80% of the starting material was ring opened. In turn, the ring opened material is made of 75% of the same four conformers observed at physiological pH, showing the same 'structure lines' described above. The remaining 25% is made of two different derivatives containing N-methyl groups (FIGURE 1). It cannot be excluded that one of these derivatives is the deformylated compound observed by HPLC and mass spectrometry[2,17] . The analysis of the structure of these products, however, is beyond the objectives of the present study.

3) We found that slow ring fission of m7Guo also occurs in wet DMSO and that the % of ring opening depends on the amount of water present. Once the water present is exhausted the carbon-13 spectrum does not show any trace of other products besides the starting material and the ring opened derivative. Further ring opening was not observed even one year after the preparation of the NMR tube. We also found that in DMSO the ring opened derivative exists only in *two* instead of four conformations (see TABLE 1). No formation of aggregates was observed. Moreover, it was found that, within the experimental error and taking into account the conversion factor δ^{TMS} (DMSO) = 39.5 ppm, the chemical shifts of the two conformers present in DMSO are the same as those of reference 24.

The results of the points **1)**, **2)** and **3)** were confirmed by the following:

a) If the material ring opened at physiological pH, described in point 1), is dried and redissolved in *dry* DMSO only the *two* sets of signals described in point 3) are present in the carbon-13 spectrum. No modification of the spectrum was observed even when heating the sample up to 80° C for a few hours. No formation of aggregates was observed. However, if water is progressively added to the DMSO solution, *four* sets of signals, i.e. the same as in pure water, appear but two of them (those corresponding to the E form, see the scheme for R=Ribose) are predominant, the intensity of the other two *depending on the amount of water present.* After addition of water appearance of 'structure lines' was observed.

b) The ring opened derivative was prepared according to the procedure described in reference 1.

In water this compound shows the same features described in point 1) and in DMSO those described in point 3).

c) Cleavage of the N9-sugar bond of the derivative obtained according to reference 1 was obtained following the procedure described by the same authors. This derivative gives in water *two* sets of signals of nearly equal intensity, whose $\delta(^{13}C)$ values are reported in TABLE 1. The proton spectrum of this compound in water shows the same dynamic equilibrium described in reference 3 ($E \rightleftharpoons Z$, see the Scheme for R=H). However, according to our carbon-13 data, the conformational assignment should be reversed (see below). No formation of aggregates is observed in this case even in water. In DMSO the the derivative gave only a single set of signals, whose $\delta(^{13}C)$ values are also reported in TABLE 1. When water is progressively added to the DMSO solution, the second set of signals appears, its relative intensity depending again on the amount of water. Clearly, the ring opened derivative (R=H) gives half the isomers of the parent compound (R=Ribose) since an asymmetry center is suppressed and the product now has a NH_2 instead of a NHRib group. Moreover, the fact that the compound does not show formation of aggregates in water indicates that in the case of the parent compound (R=Ribose) the formation of aggregates is a process involving some kind of association between the NCH3CHO and the NHRib region of the molecule. This rules out the possibility that the aggregates are due to specific associations through the pyrimidine O6-N1-N2 region similar to those observed for guanosine in water[22-23] .

We were not able to obtain all the nitrogen-15 NMR signals of the ring opened derivative, either with R=H or R=Rib, and only those pertaining to N1, NH2 and N9 were observed. The $\delta(^{15}N)$ values in DMSO are the following: R=Rib: -235.0 (N1), -298.1 (NH2), -204.0 (N9); R=H: -236.3 (N1), -297.4 (N2), -301.2 (N9). For both compounds the chemical shift of N1 is same as that of the 'pyrrole-like' nitrogen of guanosine itself [25,26] . This confirms that for both compounds the oxo form is largely predominant over the tautomeric hydroxy form.

Conformers identification by carbon-13 NMR

According to a previous study[3] the dynamic equilibrium of the two conformers of the ring opened derivative (R=H) in water is due to the restricted rotation around the C8-N7 amidic bond leading to geometrical Z and E isomerism. The partial double bond character of the r.o. derivative originates from the mesomeric structure bearing a positive charge on nitrogen, the contribution of which increases with the polarity of the solvent and in water and DMSO should be quite impor tant. Our carbon-13 NMR data are in agreement with Boiteux et al.[3]. Table 1 shows that not only the carbonyl carbons of the two forms in water are in the range expected for an amide[27], but also that the chemical shift difference between the two, as well as that of the corresponding N-methyl groups, are those expected for the cisoid and transoid E and Z isomers, cf. for example, the chemical shift differences for the two forms of N-methylformamide[28] or the methyl resonances of N,N-dimethylbenzamides and related compounds[29]. In the E form the N-CH3 group is gamma-*cis* to the oxygen atom, thus a mutual *upfield* steric shift of the N-methyl and the formyl carbons is expected compared to the Z form. The comparison of the carbon-13 chemical shifts for R=H in dimethylsulfoxide with those in water (TABLE 1) shows that the one present in DMSO is the E form, i.e. that the assignment previously made should be reversed. This conclusion is in agreement with the fact that disubstituted formamides exist predominantly in the conformation that has the larger group in the transoid position to the carbonyl oxygen[30,31]. Moreover, in the present case the E is stabilized compared to the Z form, not only by the favourable steric arrangement of the more bulky substituents, but also by the intramolecular hydrogen bond which can be formed between the formyl proton and the oxygen atom at C6. This leads to the formation of a pseudo-cyclic six-membered ring structure capable of increasing the barrier to rotation by several kJ/mol[31]. The existence of a strong intramolecular hydrogen bond explains why the E form in DMSO does not interconvert into the Z even at 70-80 °C. In water specific solvent-amide interactions[32], due to extensive *inter*molecular H-bonding with the solvent, overwhelm the stabilization energy due to the *intra*molecular H-bond of the E form, and make the energy difference between the E and Z forms much smaller than in DMSO. Accordingly, in water the E isomer, which is always the first to be formed, interconverts into the Z isomer at room temperature, and a nearly 1:1 equilibrium of the E and Z forms is attained in about one hour.

When R = Ribose, the conformational equilibrium around the amidic bond should be only slightly affected compared to R=H. In agreement with this, the ^{13}C chemical shifts for R=Ribose are nearly the same as those for R = H, *cf.* in particular the signals for the formyl and the N-methyl carbons (TABLE 1). Again, in DMSO only the E form is present, while in water both the E and Z forms are present in nearly the same concentration. Also, no interconversion of the E into the Z form is observed in DMSO even at 70-80 °C, while in water the time required for R = Ribose to attain the nearly 1:1 equilibrium of the two forms is virtually the same as that for R = H, as can easily be verified by proton NMR.

However, the presence of ribose and consequently of a further 'asymmetry' center at N9 leads to the existence of two more conformational isomers. Consequently, the N-CH3 and N-CHO resonances of the E and Z isomers are splitted by 0.1-0.3 ppm, as shown in TABLE 1. This is likely to be due to the restricted rotation around the C4-N9 bond, although hindered nitrogen pyramidal inversion of N9 (whose barrier would possibly be increased by some kind of hydrogen bonding) cannot be excluded. Anyway, the isomers already described in DMSO[24] are the two isomers of the E form for R=Ribose in DMSO and not rotational isomers around the C5-N7 bond.

DISCUSSION

The results presented here show that at physiological pH 7-methylguanosine undergoes spontaneous imidazole ring opening and formation of the formamido pyrimidine derivative reported in SCHEME 2.

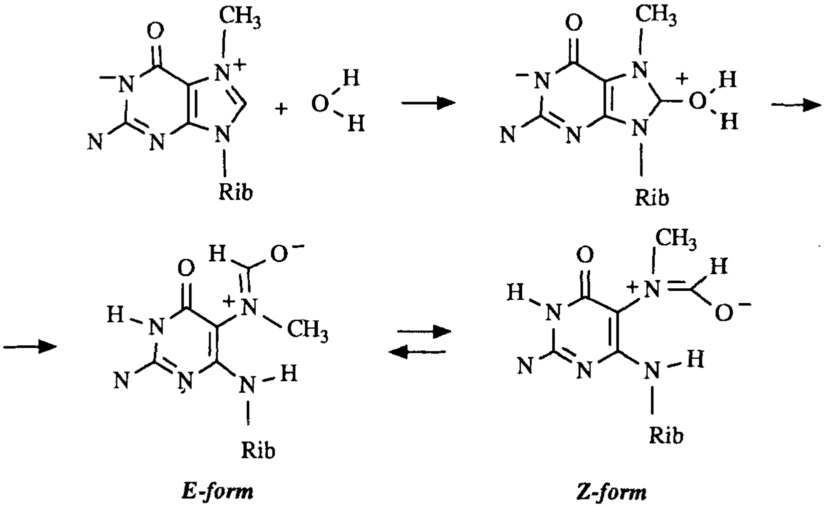
It appears that the ambiguities concerning the number and the structure of 7-methylguanosine ring opened derivatives[2,4,7,17] are related, on one side, to the strongly basic conditions used to obtain the imidazole ring scission in vitro, and, on the other, to the conformational flexibility of the r.o. derivative in water. The r.o. formamido pyrimidine derivative of 7-methylguanosine exists in water in *four* different conformations in slow equilibrium in the NMR scale: two arising from the restricted rotation around the N-CHO bond (E and Z, reported in SCHEME 2) and the other two from the restricted rotation around the C-NHRib bond (not reported in the scheme). Moreover, each conformation is capable of giving different kinds of *intra-* and *inter*-molecular hydrogen bonds and formation of aggregates.

Our data show that, contrary to previous assumptions[21], the ring opening reaction is much faster than the hydrolysis of the glycosylic bond. On the other hand, removal of the sugar decreases the number of conformers, which are now only the E and Z ones arising from the hindered rotation of the amidic bond, in agreement with the findings of Boiteux et al.[3].

Our results are in agreement with an early study reporting that spontaneous ring fission does occur in 7-methylguanine DNA residues under physiological conditions[33] in vitro, and also with more recent investigations showing that the N7 guanine adducts with the hepatocarcinogen aflatoxin B1, formed in rats liver, undergo ring scission under physiological conditions[34].

As recalled above, the conversion of 7-methylguanine to the ring opened formamidopyrimidine derivative creates potentially lethal lesions in cellular DNA[10]. There is evidence that these lesions are enzymatically removed[11]. Clearly, the conformational flexibility and the hydrogen bonding capabilities of the ring opened formamido pyrimidine derivative should be taken into account in

SCHEME 2



Nucleophilic attack of a water molecule on the imidazole ring of 7-methylguanosine amd formation of a conformationally mobile formamidopyrimidine derivative.

any investigation dealing with the mutual recognition mechanism between this species and the specific enzyme active in repairing such kind of lesions[11].

It is worth noting that the chemical behaviour of 7-methylguanosine, which undergoes spontaneous imidazole ring fission in water at physiological pH, is very different from that of its unmethylated counterpart, in which the ring fission of the C8-N9 bond requires use of OH· and H· radicals, generated by water radiolysis[12]. Clearly, the decrease of electronic charge in the imidazole ring due to methylation at N7 (or to the formation of N7 adducts) makes this region of the molecule more suitable to attack by a nucleophilic water molecule. On the other hand, the hydrolysis of 7-methylguanosine is a rather slow reaction having $\tau_{1/2}$ = 85h at 25°C. Assuming, as is the case of most pseudo first-order reactions, that $\tau_{1/2}$ is halved every 10 degrees increase in temperature, the imidazole ring of 7-methylguanosine requires about 40 hours to be 50% open in physiological conditions. In a system where water is not freely accessible (cf. our results in wet dimethylsulfoxide) the reaction becomes even slower, since its rate is related to the probability that one molecule of m7Guo encounters one molecule of water. This suggests that one of the reasons for the conflicting results about the presence of ring opened formamido pyrimidine derivatives in the liver DNA of rats given methylating carcinogenic agents[14-16] might be the different degree of hydrolysis of 7-methylguanine in neutral conditions. Since the hydrolysis is a slow reaction, in some cases enzymatical removal of the methylated base may have occurred before the ring opening has reached a measurable extent.

A final remark concerns 7-methylguanosine as a component of the 5'-terminus of several mRNA's of eukaryotic cells. Some authors[6] report that the 7-methylguanosine component is present in the *deprotonated* and some others[5,35] in the *protonated* form. However, at physiological pH it should be present in a nearly 1:1 equilibrium of the two forms (as shown in SCHEME 1), since 7-methylguanosine has a pK=7.2[1]. If, as is generally the case, the lifetime of eukariotic mRNA's is much shorter than $\tau_{1/2}$ for ring opening, hydrolysis of m7Guo has not to be taken into account. But even when $\tau_{1/2}$ is of the same order as the timelife of eukaryotic mRNA's it is likely that hydroylysis of m7Guo does not occur. Indeed, there is some evidence that the 7-methylguanosine component is located in a water-free region. According to the intercalating model for the $m^7G^{5'}$ppp5'AmpA segment of the mRNA 5' terminus[36], the 'Amp- and $m^7G^{5'}$p-parts '*are in close vicinity creating hydrophobic interactions and a water-free channel*' (in the author's own words).

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